PTO-1390 (Modified) (2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
522-1778	

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR 1.5)	
10/049868	bc X54

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/07874	August 8, 2000	August 10, 1999

TITLE OF INVENTION

Cell Lines, ligands and antibody fragments for use in pharmaceutical compositions for preventing and treating haemostasis disorders

APPLICANT(S) FOR DO/EO/US

Hans Deckmyn and Nancy Cauwenberghs

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31)
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau)
   b. ☒ has been communicated by the International Bureau
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau)
   b. ☒ have been communicated by the International Bureau
   c. ☐ have not been made, however, the time limit for making such amendments has NOT expired
   d. ☐ have not been made and will not be made
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3))
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4))
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5))
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409)
12. ☐ A copy of the International Search Report (PCT/ISA/210)

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
15. ☒ A **FIRST** preliminary amendment
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment
17. ☐ A substitute specification
18. ☐ A change of power of attorney and/or address letter
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4)
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4)
22. ☐ Certificate of Mailing by Express Mail
23. ☐ Other items or information

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR 1 5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/049868	PCT/EP00/07874	522-1778

24. The following fees are submitted	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☐ Neither international preliminary examination fee (37 CFR 1 482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
☐ International preliminary examination fee (37 CFR 1 482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
☐ International preliminary examination fee (37 CFR 1 482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
☐ International preliminary examination fee (37 CFR 1 482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	21 - 20 =	1	x $18 00	**$18.00**	
Independent claims	9 - 3 =	6	x $84.00	**$504.00**	
Multiple Dependent Claims **(check if applicable)**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,412.00**	
☒ Applicant claims small entity status See 37 CFR 1 27) The fees indicated above are reduced by 1/2				**$706.00**	
SUBTOTAL =				**$706.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1 492 (f)) +				**$0.00**	
TOTAL NATIONAL FEE =				**$706.00**	
Fee for recording the enclosed assignment (37 CFR 1 21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$706.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$706.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No _____ in the amount of _____ to cover the above fees A duplicate copy of this sheet is enclosed

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No **12-0913** A duplicate copy of this sheet is enclosed

d. ☐ Fees are to be charged to a credit card **WARNING:** Information on this form may become public **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

William M. Lee, Jr.

NAME

26,935

REGISTRATION NUMBER

FEBRUARY 11, 2002

DATE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE THE APPLICATION OF)
)
Deckmyn et al)
)
SERIAL NO.: To be assigned)
)
FILED: Herewith)
)
FOR: CELL LINES, LIGANDS AND)
ANTIBODY FRAGMENTS FOR USE IN)
PHARMACEUTICAL COMPOSITIONS)
FOR PREVENTING AND TREATING)
HAEMOSTASIS DISORDERS

AMENDMENT ACCOMPANYING APPLICATION

Honorable Director of Patents
and Trademarks
Washington, D.C. 20231

Dear Sir:

The present Application is the National Filing of International Application number PCT/EP00/07874. Appended hereto is a copy of the International Preliminary Examination report for that Application, having appended thereto the claims as they currently appear in the International Application. Before calculation of the National Filing fee for the United States, it is requested that the Application be amended as follows:

IN THE CLAIMS:

Cancel claims 1 through 43 without prejudice, and substitute new claims 44 through 64 as follows:

44. A cell line being able to produce a monoclonal antibody comprising a F_{ab} fragment which binds *in vivo* to human platelet glycoprotein GPIb, the cell line being one of the cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB, and a cell line producing a monoclonal antibody having a reactivity identical to that of a monoclonal antibody obtained from the cell line LMBP 5108CB.

45. A cell line according to claim 44, wherein the monoclonal antibody F_{ab} fragment further

prevents the binding of von Willebrand factor to human platelet glycoprotein GPIb.

46. A cell line according to claim 44 wherein the monoclonal antibody F_{ab} fragment further inhibits platelet adhesion and/or inhibits platelet activation under high shear conditions and/or inhibits platelet aggregation under high shear conditions.

47. A F_{ab} fragment, or a homologue having at least 60% amino acid sequence identity therewith, of a monoclonal antibody which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia.

48. A monoclonal antibody F_{ab} fragment or a homologue thereof according to claim 47, which prevents the binding of von Willebrand factor to human platelet glycoprotein GPIb.

49. A monoclonal antibody F_{ab} fragment or a homologue thereof according to claim 47 which further inhibits platelet adhesion and/or inhibits platelet activation under high shear conditions and/or inhibits platelet aggregation under high shear conditions.

50. A monoclonal antibody comprising a F_{ab} fragment or a homologue of the Fab fragment having at least 60% amino acid sequence identity therewith, which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia.

51. A monoclonal antibody according to claim 50 obtainable from a cell line being able to produce a monoclonal antibody comprising a F_{ab} fragment which binds *in vivo* to human platelet glycoprotein GPIb

52. A monoclonal antibody according to claim 50, being the murine monoclonal antibody 6B4.

53. A humanized monoclonal antibody derivable from the cell line of claim 44.

54. A pharmaceutical composition comprising a monoclonal antibody F_{ab} fragment or a homologue thereof having at least 60% amino acid sequence identity therewith, of a monoclonal antibody which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia.

55. A method of treatment and/or prevention of a haemostasis disorder comprising administering to a patient in need thereof a therapeutically effective amount of a monoclonal antibody F_{ab} fragment or a homologue having at least 60% amino acid sequence identity therewith, of a monoclonal antibody which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia.

56. A method of treatment and/or prevention according to claim 55, wherein the therapeutically effective amount ranges from 80 μg/kg to 4 mg/kg.

57. A method according to claim 55, comprising further administration of at least a thrombolytic agent.

58. A method according to claim 55 comprising further administration of at least a thrombolytic agent wherein the thrombolytic agent is selected from aspirin, heparin, tissue plasminogen activators, streptokinase, reptilase and staphilokinase.

59. A polynucleotide encoding for an antigen-binding monoclonal antibody F_{ab} fragment or a homologue thereof having at least 60% amino acid sequence identity therewith, of a monoclonal antibody which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia.

60. A DNA probe for detecting the polynucleotide sequence of claim 59, comprising a nucleic acid molecule having a sequence complementary to the coding sequence of said polynucleotide.

61. A polynucleotide sequence as shown in SEQ.N°1.

62. A polynucleotide sequence as shown in SEQ.N°2.

63. An amino acid sequence as shown in SEQ.N°3.

64. An amino acid sequence as shown in SEQ.N°4

REMARKS

The above Amendments are being made in order to introduce a new claim set in the U.S. Application, as well as eliminate multiple dependancy should any multiple dependency remain, that is unintended, and the Patent and Trademark Office is requested to cancel any multiple dependant claims without prejudice before calculation of the Application filing fee.

Examination of the Application on its merits is awaited.

Respectfully submitted,

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CELL LINES, LIGANDS AND ANTIBODY FRAGMENTS FOR USE IN PHARMACEUTICAL COMPOSITIONS FOR PREVENTING AND TREATING HAEMOSTASIS DISORDERS.

The present invention relates to novel cell lines and to ligands, namely human and/or humanized monoclonal antibodies, as well as fragments such as Fab or single variable domains and derivatives and combinations thereof, obtainable from the said cell line. It also relates to pharmaceutical compositions comprising said ligands or antibody fragments and to methods of preventing and treating haemostasis disorders, in particular antithrombotic treatments in humans, by administration of the said ligands or antibody fragments to patients in need thereof. It further relates to a polynucleotide encoding for the antigen-binding Fab fragment of a monoclonal antibody derivable from the said cell line.

BACKGROUND OF THE INVENTION

The coagulation of blood involves a cascading series of reactions leading to the formation of fibrin. The coagulation cascade consists of two overlapping pathways required for hemostasis. The intrinsic pathway comprises protein factors present in circulating blood, while the extrinsic pathway requires tissue factor which is expressed on the cell surface of a variety of tissues in response to vascular injury. Agents interfering with the coagulation cascade, such as heparin and coumarin derivatives, have well known therapeutic uses in the prophylaxis of venous thrombosis.

Aspirin also provides a protective effect against thrombosis. It induces a long-lasting functional defect in platelets, detectable clinically as a prolongation of the bleeding time, through inhibition of the cyclooxygenase activity of the human platelet enzyme prostaglandin H-synthase (PGHS-1) with doses as low as 30 to 75 mg. Since gastrointestinal side effects of aspirin appear to be dose-dependent, and for secondary prevention, treatment with aspirin is recommended for an indefinite period, there are practical reasons to choose the lowest effective dose. Further it has been speculated that a low dose (30 mg daily) might be more antithrombotic but attempts to identify the optimal dosage have yielded conflicting results. It has been claimed that the dose of aspirin needed to suppress fully platelet aggregation may be higher in patients

with cerebrovascular disease than in healthy subjects and may vary from time to time in the same patient. However, aspirin in any daily dose of 30 mg or higher reduces the risk of major vascular events by 20 % at most, which leaves much room for improvement. Further, the inhibiting role of aspirin may lead to prevention of thrombosis and to excess bleeding. The balance between the two depends critically on the absolute thrombotic versus hemorrhage risk of the patient.

In patients with acute myocardial infarction, reduction of infarct size, preservation of ventricular function and reduction in mortality has been demonstrated with various thrombolytic agents. However these agents still have significant shortcomings, including the need for large therapeutic doses, limited fibrin specificity, and significant associated bleeding tendency. Recombinant tissue plasminogen activator (t-PA) restores complete patency in just over one half of patients, whereas streptokinase achieves this goal in less than one third. Further, reocclusion after thrombolytic therapy occurs in 5 to 10 % of cases during the hospital stay and in up to 30 % within the first year according to Verheugt et al., *J. Am.Coll.Cardiol.* (1996) 27:618-627. Numerous studies have examined the effects of adjunctive antithrombin therapy for patients with acute myocardial infarction. For instance, U.S.Patent 5,589,173 discloses a method for dissolving and preventing reformation of an occluding thrombus comprising administering a tissue factor protein antagonist, such as a monoclonal or polyclonal antibody, in adjunction to a thrombolytic agent.

In arterial blood flow, the platelet adhesion is mainly supported by the platelet receptor glycoprotein (GP) Ib which interacts with von Willebrand factor (vWF) at the site of vessel wall injury. Blood platelets, through the processes of adhesion, activation, shape change, release reaction and aggregation, form the first line of defence when blood vessels are damaged. They form a hemostatic plug at the site of injury to prevent excessive blood loss. Extensive platelet activation however may overcome the normal thrombo-regulatory mechanisms that limit the size of the hemostatic plug. Platelets then become major prothrombotic offenders predisposing to vaso-occlusive disease.

The formation of a platelet plug during primary haemostasis and of an occluding thrombus in arterial thrombosis involves common pathways. The first

event is platelet adhesion to subendothelial collagen, exposed upon vessel injury, which can be a ruptured atherosclerotic plaque. Circulating vWF binds to the collagen and, under the influence of high shear stress, undergoes a conformational change allowing it to bind to its receptor, GPIb/IX/V, on the platelet membrane. This interaction is essential in order to produce a thrombus, at least in smaller vessels or stenosed arteries where shear stress is high, and results in slowing down the progress of the platelets across the damaged surface. Full immobilisation of platelets occurs when collagen binds to its receptor GPIa/IIa (integrin $\alpha_2\beta_1$). In addition, collagen activates platelets mainly by binding to GPVI, another collagen receptor. When platelets are activated, GPIIb/IIIa (integrin $\alpha_{IIB}\beta_3$) undergoes a conformational change and acquires the ability to bind to fibrinogen and vWF which crosslink adjacent platelets to finally form platelet aggregates.

Lately much effort has been directed to develop antibodies and peptides that can block the binding of the adhesive proteins to GPIIb/IIIa and many of these are being tested in clinical trials. One approach to blocking platelet aggregation involves monoclonal antibodies specific for GPIIb/IIIa receptors. Specifically, a murine monoclonal antibody named 7 E3 useful in the treatment of human thrombotic diseases is described in EP-A-206,532 and U.S.Patent 5,387,413. However it is known in the art that murine antibodies have characteristics which may severely limit their use in human therapy. As foreign proteins, they may elicit an anti-immunoglobulin response termed human anti-mouse antibody (HAMA) that reduces or destroys their therapeutic efficacy and/or provokes allergic or hypersensitivity reactions in patients, as taught by Jaffers et al., *Transplantation* (1986) 41:572. The need for re-administration in therapies of thromboembolic disorders increases the likelihood of such immune reactions. While the use of human monoclonal antibodies would address this limitation, it has proven difficult to generate large amounts of such antibodies by conventional hybridoma technology.

Recombinant technology has therefore been used to construct "humanized" antibodies that maintain the high binding affinity of murine monoclonal antibodies but exhibit reduced immunogenicity in humans. In particular, there have been suggested chimeric antibodies in which the

variable region (V) of a non-human antibody is combined with the constant (C) region of a human antibody. As an example, the murine Fc fragment was removed from 7E3 and replaced by the human constant immunoglobulin G region to form a chimera known as c7E3 Fab or abciximab. Obtention of such chimeric immunoglobulins is described in detail in U.S.Patent 5,770,198.

The potential for synergism between GPIIb/IIIa inhibition by monoclonal antibody 7E3 Fab and thrombolytic therapy was evaluated by Kleiman et al., *J.Am.Coll.Cardiol* (1993) 22:381-389. Major bleeding was frequent in this study. Hence, the potential for life-threatening bleeding is clearly a major concern with this combination of powerful anti-thrombotic compounds.

The GPIb-vWF axis therefore presents an attractive alternative to GPIIb/IIIa-fibrinogen as a target for platelet inhibition, since a suitable inhibitor might be expected to down regulate other manifestations of platelet activity such as granule release, thought to play a role in the development of arteriosclerosis. Activation of platelets is accompanied by secretion of vasoactive substances (thromboxane A2, serotonin) as well as growth factors such as PGDF. Therefore, early inhibition of platelet activation and hence prevention of the secretion of their growth and migration factors, via a GPIb blocker, would reduce the proliferation of smooth muscle cells and restenosis after thrombolytic therapy. Moreover, the interaction of GPIb with the damaged vessel wall (adhesion, as well as aggregation and secretion of platelet content) is highly blood flow dependent. Unlike GPIIb/IIIa interactions, GPIb-vWF interaction occurs exclusively under the high flow conditions, as occurs in small arteries or created by arterial stenoses. Hence, GPIb inhibition represents theoretically an ideal way to target effective platelet inhibition to damaged arterial areas. GPIb inhibition therefore appears particularly suited to treat patients with acute coronary syndromes, transient cerebral attacks and claudication due to peripheral arterial diseases, including prevention of the frequently letal thrombotic complications of acute coronary syndromes, angioplasty, unstable angina and myocardial infarction.

Despite these potential advantages, the development of compounds that interfere with the vWF-GPIb axis has lagged behind. Only a few *in vivo* studies described the effects of inhibition of platelet adhesion on

thrombogenesis. They include the use of anti-vWF monoclonal antibodies, GPIb binding snake venom proteins like echicetin and crotalin, aurin tricarboxylic acid that binds to vWF and recombinant vWF fragments like VCL, all of which inhibit vWF-GPIb interaction. All these molecules were anti-thrombotic, particularly in studies where a thrombus was formed under high shear conditions. U.S.Patent 5,486,361 discloses a monoclonal antibody 4H12 which specifically binds to the α chain of GPIb and, by means of this interaction, totally inhibits the binding of thrombin to normal human platelets. In addition, it inhibits more than 90% of thrombin-induced von Willebrand factor or fibrinogen binding to platelets. Further, 4H12 does not inhibit ristocetin- or botrocetin-induced binding of von Willebrand factor to platelet cells, which indicates that this antibody does not prevent von Willebrand factor binding to GPIb. A number of potent inhibitory anti-GPIb antibodies, such as LJIb1 disclosed by F.Pareti et al. in *British Journal of Haematology* (1992) 82, 81-86, have been produced and were extensively tested with respect to their *in vitro* effect under both static (platelet agglutination, vWF-binding) and flow conditions. However for none of these anti-human GPIb antibodies an *in vivo* anti-thrombotic effect could be demonstrated. *In vivo* data obtained by B.Becker and J.L.Miller (*Blood* (1989)2:680-694) describe the effect of injecting guinea pigs with intact antibody or $F(ab')_2$ fragments of PG1, a monoclonal anti-guinea pig GPIb antibody. After intraperitoneal injection of the intact antibody, a hemorrhagic state was produced with a significant lengthening of the bleeding time and drop of the platelet count to 50% of its baseline value. Injection of 0.63 to 2.5 mg/kg of the $F(ab')_2$ fragments did not decrease the platelet count more than 21%, and bleeding times never increased by more than one minute over baseline values. However, in this particular study the antithrombotic effect of the $F(ab')_2$ fragments was not further investigated by e.g. testing the fragments in an animal thrombosis model. In a follow-up study J.L.Miller et al., *Arterioscler.Thromb.* (1991) 11:1231-6 disclosed that the $F(ab')_2$ fragments of PG1 in guinea pigs using these could effectively reduce thrombus formation on a laser-induced injury. Unfortunately, this antibody does not cross react with human platelets and therefore it has no further clinical relevance for human therapy.

Part of this rather surprising lack of *in vivo* studies is due to the low cross reactivity of the anti-human GPIb monoclonal antibodies with platelets from commonly used laboratory animals. This predisposes to the use of non-human primates as experimental animals. However, even then attempts to perform *in vivo* studies are hampered because injection of the anti-GPIb monoclonal antibodies, as well as the snake venom protein echicetin that reacts with GPIb, invariably causes severe thrombocytopenia, as taught by US-A-5,336,667. WO-A-002667 further discloses monoclonal antibodies F_{ab} fragments but does not discuss thrombocytopenia and does not mention *in vivo* tests.

One persistent concern with all available thrombolytic and anti-thrombotic agents, including aspirin, is to induce a risk of overdose and therefore of excessive and life-threatening bleeding. Therefore a first goal of the present invention is to provide a thrombus formation protective means by providing a platelet adhesion inhibitor that does not induce a risk of bleeding. A second goal of the present invention is to provide a thrombus formation protective means by providing an inhibitor of platelet adhesion without incurring the risk of thrombocytopenia. A third goal of the present invention is the targetting of platelet adhesion, activation and aggregation under high shear conditions, which is of particular importance in the setting of highly stenotic atherosclerotic lesions. The specific targetting of highly stenotic areas in the circulation should make GPIb inhibition particularly suitable for treating unstable angina and in the chronic prevention of arterial occlusion. Unlike with GPIIb/IIIa inhibition, platelet aggregation as well as hemostasis is not expected to be inhibited in low shear vessels, i.e. in veins and normal arteries. Bleeding complications from these vessels by inhibition of GPIb may therefore be expected to be better reduced than with GPIIb/IIIa inhibition.

SUMMARY OF THE INVENTION

The essence of this invention is that by using a ligand such as a monovalent Fab fragment of a certain inhibitory human GPIb antibody, a marked prevention of platelet dependent thrombus formation targetted to high shear flow vessels and without incurring thrombocytopenia can be obtained. Moreover, this is so far the only anti-human GPIb monoclonal antibody for

which the antithrombotic efficacy has been proven *in vivo* in an animal thrombosis model.

The present invention therefore first includes a cell-line deposited with the Belgian Coordinated Collections of Micro-organisms, under accession number LMBP 5108CB. Secondly the present invention includes a ligand which binds to the human platelet glycoprotein GPIb and prevents the binding of von Willebrand factor (vWF) to GPIb and which preferably does not produce thrombocytopenia when administered to a primate (in this invention, the word "primate" also relates to humans) at a dose of up to at least 4 mg/kg by bolus intravenous administration. In particular the present invention includes a ligand derived from a monoclonal antibody such as 6B4 obtainable from the said cell line. Thirdly the present invention relates to an antigen-binding Fab fragment, or a homolog or derivative of such fragment (including a humanized fragment which might be divalent, trivalent or tetravalent), which may be obtained by proteolytic digestion of the said monoclonal antibody by papain, using methods well known in the art. Fourthly the present invention includes pharmaceutical compositions comprising said ligands or fragments which are useful for preventing and treating haemostasis disorders, in particular for anti-thrombotic treatments, in humans. Finally the present invention includes polynucleotide sequences encoding for the above-mentioned monoclonal antibodies or Fab fragments thereof. It will be appreciated that a multitude of nucleotide sequences fall under the scope of the present invention as a result of the redundancy in the genetic code. The present invention also includes nucleic acid molecules comprising sequences which are complementary to the coding sequence of said polynucleotides and the use of such molecules as DNA probes for detecting the said polynucleotides.

The present invention is first based on the observation of the antithrombotic effect of human platelet glycoprotein GPIb blocking monoclonal antibody 6B4 Fab fragment derived from the cell line LMBP 5108CB in a baboon model of arterial thrombosis. Two *in vivo* models were used and described in this invention: the first model is an arteriovenous shunt model in which an extracorporeal loop is made between the femoral artery and the femoral vein. Within this loop a collagenic graft is incorporated and the platelet

deposition onto this graft is measured, as shown in examples 7-8 and figures 5-6. Baboons were either pre-treated with said Fab fragment to study the effect on platelet deposition on a thrombogenic device, or treated 6 minutes after placement of the thrombogenic device in order to investigate the effect on inter-platelet cohesion. In this first study, it was observed that blockade of GPIb had no effect on platelet deposition onto a fresh thrombus, whereas pre-treatment effectively reduced thrombus formation. The second model is a clinically even more relevant model mimicking platelet-mediated thrombotic occlusion as occuring in stenosed and intimally damaged coronary arteries *in vivo*. In this second model a stenosis is applied to a damaged femoral artery, and blood flow is measured. Due to platelet aggregate formation the stenotic area occludes but reopens due to embolisation, resulting in regular cyclic flow reductions as shown in example 9.

Secondly, the present invention is based on *in vitro* and *in vivo* studies of the antithrombotic efficacy of the monoclonal antibody, 6B4 (IgG1), raised against human platelet glycoprotein Ib. *In vitro*, 6B4 potently inhibits the binding of vWF to human GPIb both under static and flow conditions, as further illustrated by the following examples, and it also binds to baboon platelets. When 6B4 was injected into baboons, both the intact monoclonal antibody and its $F(ab')_2$ fragments caused immediate and severe thrombocytopenia, whereas Fab fragments of 6B4 did not. Furthermore, Fab fragments studied in the two baboon models effectively prevented platelet-dependent arterial thrombosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the inhibiting effect of 6B4 Fab fragments on the ristocetin- and botrocetin-induced binding of vWF to rGPIb.

Figure 2 shows the inhibiting effect of 6B4 Fab fragments on platelet adhesion to collagen type I under flow.

Figure 3 shows binding curves of 6B4 and its fragments to baboon platelets in plasma.

Figure 4 shows the inhibitory effect of 6B4 and its fragments on ristocetin-induced baboon platelet aggregation.

Figure 5 shows platelet adhesion and deposition onto three

thrombogenic devices placed in baboons either untreated (fig. 5A) or treated (fig. 5B) with 6B4 Fab fragments.

Figure 6 shows the influence of late treatment of baboons with 6B4 Fab fragments on platelet deposition.

Figure 7 shows the effect of 6B4 Fab fragments on cyclic flow reductions.

Figure 8 shows the effect of 6B4 Fab fragments on platelet count.

Figure 9 shows the effect of 6B4 Fab fragments on bleeding time.

Figure 10 shows the inhibition of *ex vivo* platelet aggregation by 6B4 Fab fragments.

Figure 11 shows the occupancy of GPIb receptors by 6B4 Fab fragments.

Figure 12 shows (lower lines) the amino acid sequence (SEQ.No.3) and (upper lines) the nucleotide sequence (SEQ.No.1) for the variable regions VL of the light chains of the 6B4 monoclonal antibody.

Figure 13 shows (lower lines) the amino acid sequence (SEQ.No.4) and (upper lines) the nucleotide sequence (SEQ.No.2) for the variable regions VH of the heavy chains of the 6B4 monoclonal antibody.

DEFINITIONS

The term "antibody" refers to intact molecules as well as fragments thereof, which are capable of binding to the epitope determinant of the relevant factor or domain of the factor.

"Humanized antibody" as used herein, refers to antibody molecules in which amino acids have been replaced in the non-antigen binding regions in order to more closely resemble a human antibody.

The term "homolog" as used herein with reference to ligands in accordance with the present invention refers to a molecule which will compete with or inhibit binding of one of the ligands in accordance with the present invention to the target site. The binding should be specific, i.e. the binding of the alternative molecule should be as specific to the site as the ligand in accordance with the present invention. Where the ligands in accordance with the present invention include amino acid sequences, homology may include having at least about 60%, preferably at least 80%, more preferably at least

90% and most preferably at least 95% amino acid sequence identity with the relevant ligand.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will be described with reference to certain embodiments and figures but the present invention is not limited thereto but only by the following claims.

The present invention provides a cell-line deposited with the Belgian Coordinated Collections of Micro-organisms, under accession number LMBP 5108CB. The present invention further provides cell lines producing monoclonal antibodies having a reactivity, namely a reactivity towards human GP Ib, substantially identical to that of monoclonal antibodies obtainable or obtained from cell line LMBP 5108CB, as well as the human monoclonal antibodies obtainable from the said further cell lines.

The present invention also provides ligands which are able to bind to the human platelet glycoprotein GPIb and also preferably able to prevent the binding of von Willebrand factor (vWF) to GPIb, in particular ligands derived from a monoclonal antibody (referred to as 6B4) obtainable from said cell line LMBP 5108CB or from equivalent cell lines such as above defined. More preferably, such a ligand should be able to recognize an epitope located on human platelet glycoprotein GPIb. For instance, the present invention relates to ligands of the above-mentioned type, being derived from a monoclonal antibody produced by on purpose immunization in animals. The present invention also provides an antigen-binding Fab fragment, or a homolog or derivative of such fragment, which may be obtained by proteolytic digestion of the said monoclonal antibody by papain, using methods well known in the art. In order to reduce the immunogenicity of the murine anti-GPIb monoclonal antibody 6B4, the present invention also includes the construction of a chimeric antibody, preferentially as a single-chain variable domain which combines the variable region of the mouse antibody with a human antibody constant region - a so-called humanized monoclonal antibody. The monoclonal antibodies produced in animals may be humanized, for instance by associating the binding complementarity determining region ("CDR") from the non-human monoclonal antibody with human framework regions - in particular the constant

C region of human gene - such as disclosed by Jones et al. in *Nature* (1986) 321:522 or Riechmann in *Nature* (1988) 332:323, or otherwise hybridized.

This invention also provides using a ligand or a humanized or hybridized monoclonal antibody or an antigen-binding Fab fragment such as specified hereinbefore as a medicament. Although aspirin will continue to be widely used for patients with vascular disease, however there are a number of situations in which increased thrombotic risk requires the use of a more potent platelet inhibitor than aspirin. Conditions such as angioplasty, coronary stenting and thrombolysis are likely to require more potent platelet inhibitors. In these acute clinical situations, the fibrous cap over an atherosclerotic plaque has been ruptured which produces deep arterial injury and exposes a much more thrombogenic surface. Furthermore, high shear forces acting on platelets passing through severely narrowed stenoses can also overcome the inhibitory effects of aspirin. Therefore a GPIb antagonist according to the invention may be used for reducing the problems of occlusion and restenosis in patients undergoing angioplasty or for the prevention of reocclusion after succesful thrombolysis by tissue plasminogen activators, streptokinase or the like. It is believed that platelet activation, as a result of the platelet adhesion, is a key component in the failure of thrombolysis. Therefore a therapeutic approach towards blocking the GPIb-vWF interaction, that results in a down-regulation of platelet signalling, represents a new way of interfering in thrombus formation.

The present invention therefore further provides pharmaceutical compositions comprising a ligand or a humanized or hybridized monoclonal antibody or an antigen-binding Fab fragment such as specified hereinbefore, in admixture with a pharmaceutically acceptable carrier. More preferably the said pharmaceutical composition comprises a human or humanized or hybridized monoclonal antibody or an antigen-binding Fab fragment thereof obtainable from the cell line LMBP 5108CB. which are useful for preventing and treating haemostasis disorders, in particular for anti-thrombotic treatments, in humans.

The use of a GPIb blocker according to the present invention is believed to be more efficient in acute situations and, in some cases, as an adjunctive therapy together with other agents such as, among others, aspirin or heparin. The pharmaceutical composition of the present invention may therefore further

comprise, in view of the so-called adjunctive therapy, a therapeutically effective amount of a thrombolytic agent. Such thrombolytic agents, as well as their usual dosage depending on the class to which they belong, are well known to those skilled in the art. Among numerous examples of thrombolytic agents which may be included in the pharmaceutical compositions of the invention, may be cited tissue plasminogen activators (t-Pa), streptokinase, reptilase, TNK-t-Pa or staphylokinase. The pharmaceutical composition should comprise the additional thrombolytic agent in a form which is suitable either for simultaneous use or for sequential use. Sequential, as used herein, means that the ligand or humanized monoclonal antibody or antigen-binding Fab fragment of the invention on the one hand and the known thrombolytic agent are administered to the patient in alternance but not within the same dosage unit.

Suitable pharmaceutical carriers for use in the pharmaceutical compositions of the invention are described for instance in Remington's Pharmaceutical Sciences 16th ed. (1980) and their formulation is well known to those skilled in the art. They include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents (for example phenol, sorbic acid, chlorobutanol), isotonic agents (such as sugars or sodium chloride) and the like. Additional ingredients may be included in order to control the duration of action of the monoclonal antibody or Fab fragment active ingredient in the composition. Control release compositions may thus be achieved by selecting appropriate polymer carriers such as for example polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinyl acetate copolymers, methylcellulose, carboxymethylcellulose, protamine sulfate and the like. The rate of drug release and duration of action may also be controlled by incorporating the monoclonal antibody active or Fab fragment ingredient into particles, e.g. microcapsules, of a polymeric substance such as hydrogels, polylactic acid, hydroxymethylcellulose, polymethyl methacrylate and the other above-described polymers. Such methods include colloid drug delivery systems like liposomes, microspheres, microemulsions, nanoparticles, nanocapsules and so on. Depending on the route of administration, the pharmaceutical composition comprising the active ingredient may require protective coatings.

The pharmaceutical form suitable for injectionable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation thereof. Typical carriers therefore include biocompatible aqueous buffers, ethanol, glycerol, propylene glycol, polyethylene glycol and mixtures thereof.

The pharmaceutical composition and medicament in accordance with the present invention may be provided to a patient by means well known in the art. i.e. orally, intranasally, subcutaneously, intramuscularly, intradermally, intravenously, intraarterially, parenterally or by catheterization. For the reasons stated above, they will be especially useful for the treatment and/or prevention of disorders of haemostasis and particularly for antithrombotic treatment or prevention. Therefore the present invention further provides a method of treatment and/or prevention of such disorders by administering to a patient in need thereof a therapeutically effective amount of a ligand or a humanized monoclonal antibody or an antigen-binding Fab fragment such as specified hereinbefore, optionally together with (simultaneously or sequentially) a therapeutically effective amount of a thrombolytic agent such as above described.

The present invention also provides a polynucleotide sequence encoding for the antigen-binding Fab fragment, or homolog or derivative of the monoclonal antibody derived from cell line LMBP 5108CB. The present invention also provides nucleic acid molecules comprising a sequence which is complementary to the coding sequence of the said polynucleotide and the use of such molecules as DNA probes for detecting the said polynucleotide.

The present invention is further described by the following examples which are provided for illustration purposes only. Data were tested for statistically significant difference. Data given in the text are mean ± SE. P-values < 0.05 are considered significantly different.

Example 1- preparation and purification of intact monoclonal antibody 6B4, $F(ab')_2$ and Fab fragments

6B4 (subtype IgG1), is a murine monoclonal antibody raised against purified human GPIb and obtainable from the cell line deposited with the Belgian Coordinated Collections of Micro-organisms under accession number

LMBP 5108CB. When added at saturating concentrations, monoclonal antibody 6B4 totally abolishes both ristocetin- and botrocetin-induced human platelet aggregation as well as shear-induced platelet adhesion to human collagen type I tested in a Sakariassen-type flow chamber at $2600s^{-1}$.

Hybridoma cells producing the monoclonal antibody 6B4 were grown and subsequently injected into pristane (i.e. 2,6,10,14-tetramethyldecanoic acid)-primed Balb/c mice. After 10 days ascites fluid was collected. The immunoglobulin (IgG) was extracted from the ascites using protein-A-Sepharose CL-4B (available from Pharmacia, Roosendaal, Netherlands).

In order to prepare $F(ab')_2$ fragments, the monoclonal antibody 6B4 was dialyzed overnight against a 0.1mol/l citrate buffer (pH 3.5) The antibody (200 parts) was digested by incubation with pepsin (1 part) available from Sigma (Saint-Louis, Missouri) for 1 hour at 37°C. Digestion was stopped by adding 1 volume of a 1M Tris HCl buffer (pH 9) to 10 volumes of antibody.

Monovalent Fab fragments were prepared by papain digestion as follows: a 1 volume of a 1M phosphate buffer (pH 7.3) was added to 10 volumes of the monoclonal antibody, then 1 volume papain (Sigma) was added to 25 volumes of the phosphate buffer containing monoclonal antibody, 10 mmol/l L-Cysteine HCl (Sigma) and 15 mmol/l ethylene diamine tetra acetic acid (hereinafter referred to as EDTA). After incubation for 3 hours at 37°C, digestion was stopped by adding a final concentration of 30 mmol/l freshly prepared iodoacetamide solution (Sigma), keeping the mixture in the dark at room temperature for 30 minutes.

Both $F(ab')_2$ and Fab fragments were further purified from contaminating intact IgG and Fc fragments using protein-A-Sepharose. The purified fragments were finally dialyzed against phosphate-buffered saline (hereinafter referred as PBS). Purity of the fragments was determined by sodium dodecylsulphate polyacrylamide gel electrophoresis and the protein concentration was measured using the bicinchonicic acid Protein Assay Reagent A (Pierce, Rockford, Illinois).

Example 2 – Method for determining deposition of platelets

Autologous blood platelets were labelled with ^{111}In-tropolone and imaging and quantification of the deposition of ^{111}In-platelets were done as

described by Kotze et al., *J.Nucl.Med.* (1991) 32:62-66. Briefly, image acquisition of the grafts, including proximal and distal silastic segments, was done with a Large Field of View scintillation camera fitted with a high resolution collimator. The images were stored on and analysed with a Medical Data Systems A^3 computer (Medtronic, Ann Arbor, MI) interfaced with the scintillation camera. Dynamic image acquisition, 2 minute images (128x128 byte mode), was started simultaneously with the start of blood flow through the devices. A two minute image (128x128 byte mode) of a 3 ml autologous blood sample (collected in EDTA) was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic images. Radioactivity in a region of similar size of circulating radioactivity in the proximal segment of the extension tubing was determined, and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. Platelet deposition was expressed as the total number of platelets deposited. The method to calculate this is described by Hanson et al, *Arteriosclerosis* (1985) 5:595-603.

Example 3 – receptor binding measurements

6B4, its $F(ab')_2$ or Fab fragments were labelled with Na-^{125}I (Amersham, Buckinghamshire, UK) using the Iodogen method as described by Fraker et al., *Biochem. Biophys. Res.Comm.* (1978) 80:849-857. Iodogen was purchased from Pierce (Rockford, IL). Platelet-rich baboon plasma, adjusted with autologous plasma to a count of 100,000 platelets/µl, was incubated with different concentrations of iodinated 6B4, F(ab')2 or Fab fragments for 15 minutes at room temperature. The mixture was layered onto 20% sucrose buffer (wt/vol) containing 0.1% (wt/vol) bovine serum albumin (BSA) and centrifuged for 4 min at 10,000g in Eppendorf tubes. The top fluid, including the plasma, was removed and the pellets were counted in a gamma-counter. This study was performed in duplicate on the platelet rich plasma of two baboons.

Example 4 - *In vitro* and *ex vivo* platelet aggregation measurement

The aggregation of platelets in response to ristocetin (1.5 mg/ml final concentration; abp, NY) was done on 10 ml blood collected in 1 ml of 3.2%

trisodiumcitrate. Platelet rich plasma was prepared by differential centrifugation as described by Van Wyk et al, *Thromb.Res.* (1990) 57:601-9 and the platelet count adjusted to 200,000 platelets/µl with autologous plasma. The aggregation response was measured in a Monitor IV Plus aggregometer (Helena Laboratories, Beaumont, Texas) and recorded for 5 minutes. The percent aggregation at 5 minutes was calculated as the difference in light transmission between platelet-rich and platelet-poor plasma.

In *in vitro* studies, the platelet rich plasma was preincubated for 5 minutes with serial dilutions of intact IgG 6B4, F(ab')2 or Fab fragments before aggregation was initiated. Inhibition of aggregation was calculated from the difference in the aggregation response of platelets without and with antibody or fragments. In the *ex vivo* determinations, inhibition was calculated from the difference in the aggregation response of platelets before and after treatment of the baboons.

Example 5 – measurement of plasma concentrations of 6B4, $F(ab')_2$ or Fab fragments and of bleeding time.

Plasma concentrations were measured using a sandwich enzyme-linked immunoassay (ELISA). Briefly, microtiter plates were coated overnight at 4°C with 5 µg/ml polyclonal goat anti-mouse IgG (Sigma). After blocking non-occupied binding sites with bovine serum albumin, serial dilutions of baboon plasma were added to the wells and incubated for two hours. Bound 6B4 (IgG, $F(ab')_2$ or Fab fragments) was detected by using goat anti-mouse IgG (Fab specific) conjugated to peroxidase (Sigma). Standard curves were constructed by adding known amounts of 6B4 (IgG, $F(ab')_2$ or Fab fragments) to baboon plasma.

Bleeding time was determined using the Simplate® II device (Organon Teknika,Durham, North Carolina) according to the instructions of the manufacturer, the volar surface of the forearm of the baboons being shaved and a pressure cuff being applied and inflated to 40 mm Hg.

Example 6 - *In vitro* effect of monoclonal antibody 6B4 and Fab fragments on binding of vWF to human GPIb under static and flow conditions

Monoclonal antibody 6B4 binds to a (1-289) recombinant (r)GPIbα fragment expressed by Chinese hamster ovary cells obtained from Meyer et al.

J. Biol. Chem. (1993) 268:20555-20562, indicating that its epitope is localized within the aminoterminal region of GPIbα.

Monoclonal antibody 6B4 Fab fragments were further tested for inhibition of ristocetin- and botrocetin-induced binding of vWF to the rGPIbα fragment using an ELISA set-up, as described by Vanhoorelbeke et al. *Thromb. Haemost.* (2000):83:107-113. Microtiter plates were coated with 5 µg/ml monoclonal antibody 2D4 for 48 hours at 4°C. Monoclonal antibody 2D4, another anti-GPIb monoclonal antibody, binds to the rGPIbα fragment but does not block vWF binding. Non-adsorbed sites were blocked with 3% skimmed milk whereafter the plates were washed with tris buffered saline (hereinafter referred as TBS) containing 0.1% Tween 20 (TBS-Tw). Purified rGPIbα fragments were immobilised on monoclonal antibody 2D4 by incubating 2µg/ml rGPIbα for 2 hours at 37 °C. After washing with TBS-Tw, increasing concentrations of 6B4 Fab fragments (diluted in TBS-Tw) were added, followed by 1.25 or 0.6 µg/ml purified human vWF (available from the red Cross Belgium), respectively when ristocetin (300 µg/mL) or botrocetin (0.5 µg/mL) were used as modulators. Binding of vWF was determined by incubating for 1 hour with HRP conjugated polyclonal anti-vWF antibody (Dako, Glostrup, Denmark), diluted 1/3000 in TBS-Tw. The color reaction, stopped with 4 mol/l H_2SO_4 was generated with orthophenylenediamine (available from Sigma). The purification of botrocetin from crude *Bothrops jararaca* venom (available from Sigma) was performed according to Fujimura et al. *Biochemistry* (1991) 30:1957-1964.

The effect of 6B4 Fab fragments on shear-induced platelet adhesion to collagen was tested in a Sakariassen-type parallel-plate flow chamber at shear rates of 650, 1,300 and 2,600 sec^{-1}, according to Harsfalvi et al. *Blood* (1995) 85:705-7011. Human collagen type I (Sigma) was dissolved in 50 mM acetic acid (1 mg/ml), dialysed for 48 hours against PBS and subsequently sprayed onto plastic Thermanox coverslips and stored at room temperature overnight before use. 12 ml of blood, anticoagulated with LMW heparin (25 U/mL, Clexane, Rhône-Poulenc Rorer, France), was preincubated with 6B4 Fab fragments at 37°C for 5 minutes and then used to perfuse the collagen-coated coverslips. After 5 minutes of perfusion, the platelets were fixed with methanol and the coverslips stained with

May-Grünwald Giemsa. Platelet adhesion (percent of total surface covered with platelets) was evaluated with a light microscope connected to an image analyser. An average of 30 fields per coverslip were analysed. Platelet adhesion was expressed as % maximal platelet adhesion obtained in the absence of inhibitor.

Monoclonal antibody 6B4 Fab fragments block the ristocetin(1 mg/ml)- and botrocetin(0.5 µg/ml)-induced human platelet agglutination with an IC_{50} of 1.2 ± 0.3 µg/ml (24 ± 6 nmol/l) and 2.0 ± 0.5 µg/mL (40 ± 10 nmol/L) respectively. 6B4 binds to an epitope localized on the aminoterminal part (His1-Val289) of GPIbα. As shown in figure 1, the 6B4 Fab fragments dose-dependently inhibited both the ristocetin- and botrocetin-induced binding of vWF to rGPIb, with an IC_{50} of 1.8 µg/ml (36 nmol/l) and 2.5 µg/ml (50 nmol/l) respectively when the binding was induced by ristocetin (300 µg/ml) or botrocetin (0.5 µg/ml).

As shown in figure 2, the 6B4 Fab fragments inhibited platelet adhesion to collagen type I in a concentration-dependent manner at shear rates of 650, 1,300 and 2,600 sec^{-1}. A 50 % reduction of surface coverage was obtained at a concentration of 3.5 µg/mL (70 nmol/l), 1.1 µg/mL (22 nmol/L) and 0.5 µg/mL (10 nmol/L) respectively for shear rates of 650, 1,300 and 2,600 sec^{-1}.

Example 7 – *in vivo* studies in baboons : Dose response effect of 6B4 Fab-fragments on platelet adhesion and deposition

Male baboons (*Papio ursinus*) weighing between 10 and 15 kg and being disease-free for at least 6 weeks were used according to procedures approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State (South Africa) and the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances (South Africa). The baboons supported permanent Teflon®-Silastic Arteriovenous (AV) shunts implanted in the femoral vessels according to Hanson et al (cited *supra*). Blood flow through the shunts varied between 100 and 120 ml/min, resulting in wall shear rates between 800 and 1,000 sec^{-1}, which compares with the shear rates found in medium sized arteries. Handling of the baboons was achieved through anaesthesia with about 10mg/kg ketamine hydrochloride (Anaket-V, Centaur Laboratory, South Africa).

In order to test the effect of the monoclonal antibody on platelet count, 6B4 and its $F(ab')_2$ and Fab fragments were administered to three different baboons. The injected dose was calculated to attain a plasma concentration of $1xKD_{50}$ i.e. the concentration needed to occupy 50% of the receptors as determined in *in vitro* experiments.

Platelet-dependent arterial thrombus formation was induced by using bovine pericardium (0.6 cm^2) fixed in buffered gluteraldehyde according to the method disclosed by Quintero et al, *J.Heart Valve Dis.* (1998) 7:262-7. The pericardium was built into the wall of silicone rubber tubing (3 mm inside diameter). The method of preparation of the thrombogenic device is described by Kotze et al, *Thromb.Haemost.* (1993) 70:672-5, except that fixed bovine pericardium instead of Dacron® vascular graft material was used. In each experiment, a thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV-shunt by means of Teflon® connectors as previously disclosed by Hanson et al (cited *supra*).

In this first approach to determine the effect of 6B4 fragments on platelet adhesion, seven baboons were used and thirteen perfusion experiments were performed. In the first five experiments (3 baboons), a thrombogenic device was placed to determine deposition of platelets according to the method of example 2. After 30 minutes, the device was removed and blood flow through the permanent AV-shunt re-established. Fifteen minutes after removal of the device, each baboon was treated with a bolus of 80 µg/kg Fab fragments of 6B4 (in 2 ml saline) and again fifteen minutes later, a second thrombogenic device was placed for 30 minutes to determine the effect of the Fab fragments on thrombogenesis. The device was again removed and blood flow through the permanent shunt established. This was followed by a second bolus injection of Fab fragments (80 µg/kg) to attain a cumulative dose of 160 µg/kg. After fifteen minutes, a third thrombogenic device was placed for 30 minutes and platelet deposition measured according to the method of example 2. In four other experiments (2 baboons) the same study protocol was used but two doses of 320µg/kg were administered.

In four other experiments (4 baboons), sham studies were performed by

using the same protocol of placement of thrombogenic devices, but the baboons were not treated with Fab fragments.

Blood was collected at different periods of time (given in the figures) to determine platelet count and haematocrit (EDTA), circulating and platelet associated radioactivity, the *ex vivo* aggregation of platelets in response to ristocetin (according to the method of example 4) and the plasma concentrations of Fab fragments (according to the method of example 5).

Example 8 – *in vivo* studies in baboons - Effect of anti-GPIb 6B4 fragments on interplatelet cohesion

In this second approach to determine the effect of 6B4 fragments on interplatelet cohesion, six baboons were selected in a manner similar to that of example 7 and used as follows. In all baboons, a thrombogenic device was placed for 24 minutes. In six experiments (3 baboons), the baboons received a bolus injection of Fab fragments of 110 µg/kg. The fragments were injected six minutes after placement of the thrombogenic device to allow enough platelets to be deposited to cover the collagen surface. In the six other experiments, the other three baboons did not receive Fab fragments.

As in example 7, blood was collected at different periods of time (given in the figures) to determine platelet count and haematocrit (EDTA), circulating and platelet associated radioactivity, the *ex vivo* aggregation of platelets in response to ristocetin (according to the method of example 4) and the plasma concentrations of Fab fragments (according to the method of example 5).

Experimental results

Figure 3 shows binding curves of anti-GPIb ^{125}I-6B4 IgG (ν), - F(ab')2 (λ) and - Fab fragments (σ) to baboon platelets in plasma. Binding of the antibody and its fragments to baboon platelets was dose-dependent and saturable: half saturation (KD_{50}) was obtained with 4.7 nmol/l, 6.4 nmol/l and 49.2 nmol/l for the monoclonal antibody 6B4 IgG, its $F(ab')_2$ and Fab fragments respectively.

Figure 4 shows the inhibitory effect of anti-GPIb 6B4 IgG (ν), - F(ab')2 (λ) and - Fab fragments (σ) on ristocetin-induced baboon platelet aggregation. When added at saturating concentrations, ristocetin-induced aggregation was completely abolished : IC_{50}-values were 4.5 nmol/l, 7.7 nmol/l and 40 nmol/l for

the monoclonal antibody 6B4 IgG, its $F(ab')_2$ and Fab fragments respectively.

When considering the effect of injection of the monoclonal antibody 6B4, $F(ab')_2$ and Fab fragments on the peripheral platelet count in baboons, the dose of the 6B4 and its fragments used were calculated, for purposes of comparison to attain a plasma concentration of $1xKD_{50}$. In one baboon, 100 µg/kg of intact antibody caused a profound decrease in the blood platelet count (< $30x10^9$pl/l) within 10 minutes after injection. After 48 hours, the platelet count was still below $100x10^9$pl/l. When 6B4 $F(ab')_2$ fragments were injected into 2 baboons, the platelet count decreased rapidly to between 120 and $150x10^9$pl/, i.e. by approximately 60%, and then reached pre-infusion values within 24 hours. Finally when 80-320 µg/kg of the monovalent 6B4 Fab fragments was injected, the platelet count (45 min after injection) decreased only by approximately 10-20% and by 26% when 640 µg/kg was injected as shown in table 1 hereinafter.

Figure 5 shows platelet deposition onto thrombogenic devices, containing bovine pericardium, placed consecutively at times 0 (λ), 60 (ν) and 120 (σ) minutes for 30 minutes (top shaded bars) for panel A and following injection of 0 (λ), 80 (ν) and 160 (σ), 320 (♦) and 640 (♥) µg/kg 6B4 Fab fragments for panel B. In the sham studies (figure 5A), placement of the previous graft had no significant effect on platelet deposition formed on subsequent grafts. In the treatment studies (figure 5B), dosages of 80 µg/kg and 160 µg/kg significantly inhibited platelet deposition in comparison to control, by approximately 43% and 53% respectively. Doses of 320 µg/kg and 640 µg/kg significantly reduced platelet deposition by 56% and 65% respectively.

Plasma levels of 6B4 Fab-fragments and inhibition of *ex vivo* agglutination determined on samples obtained 45 minutes or 2 hours after administration both changed dose- and time-dependently, as shown in table 1 hereinafter.

Bleeding times, determined in the treatment studies before and 45 minutes after injecting 80 to 320 µg/kg of 6B4 Fab fragments, were not significantly prolonged. Only a dose of 640 µg/kg significantly prolonged the bleeding time which was still less than doubled.

Figure 6 shows the influence of late treatment of baboons with 6B4 Fab fragments on platelet deposition, the thrombogenic device being placed at time 0 and platelet deposition determined for 24 minutes (top shaded bar). After six minutes (arrow), baboons were either untreated (ν) or treated with a bolus of 110 (λ) µg/kg 6B4 Fab fragments. It is thus shown that 110 µg/kg 6B4 Fab fragment did not affect platelet deposition when injected after a thrombus was allowed to form for an initial 6 minutes.

Interpretation of experimental results

The anti-GPIb monoclonal antibody 6B4, its $F(ab')_2$ and Fab fragments potently inhibited the binding of vWF to a recombinant GPIbα fragment (His1-Val289) and dose-dependently inhibited vWF-dependent human platelet agglutination. The intact monoclonal antibody and its fragments also dose-dependently inhibited human platelet adhesion to type I collagen in a flow chamber at wall shear rates of 650, 1300 and 2600 sec^{-1}. This inhibition was shear-dependent, i.e. more pronounced at higher shear.

6B4, its $F(ab')_2$ and Fab fragments also bind to and inhibit baboon platelets and inhibit baboon platelets with much the same characteristics as human platelets. As a result baboons were used for *in vivo* and *ex vivo* studies. An almost immediate, profound and irreversible thrombocytopenia developed when the intact antibody was injected into a baboon, similar to what was observed when other anti-GPIb monoclonal antibodies were injected into different experimental animals. The $F(ab')_2$ fractions also caused immediate, but reversible thrombocytopenia, but to a lesser extent than the intact antibody. The Fab fractions, on the other hand, had only a moderate effect on the blood platelet count, which strongly suggests that the Fc portion of the monoclonal antibody plays a part in the development of the irreversible thrombocytopenia.

The 6B4 Fab fractions were used to assess an anti-thrombotic effect in a baboon model of arterial thrombosis. The gluteraldehyde fixed bovine pericardium was highly thrombogenic: after 30 minutes of exposure to native flowing blood, approximately $3x10^9$ platelets deposited on the area of 0.6 cm^2. In similar studies, only approximately $0.7x10^9$ platelets accumulated on Dacron vascular graft material (0.9 cm^2) according to Kotzé et al., *Thromb. Haemost*

(1993) 70:672-675. It is therefore not surprising that a number of control thrombogenic devices occluded before 30 minutes of exposure to flowing blood.

Treatment of baboons with 6B4 Fab fragments inhibited platelet deposition on the thrombogenic devices by between 43 and 65%. The observed effect must be ascribed to the monoclonal antibody, since sequential placement of thrombogenic devices in untreated baboons caused no decreased deposition. No complete inhibition of platelet deposition was observed, even at high doses.

Example 9 - *in vivo* studies in baboons : effect of 6B4 Fab fragments on cyclic flow variations in stenosed, endothelium-injured arteries

The experimental model used herein is adapted from the model originally described by J.D.Folts et al. in *Circulation* (1982) 65:248-255 as a canine model of coronary artery stenosis with intimal damage. Basically, this model allows to study the cyclic flow reductions in coronary blood flow due to platelet-dependent thrombi forming at the site of a coronary stenosis which was created by the placement of a fixed constrictor. It provides a reproducible pattern of recurrent thrombosis to be established and is widely accepted as very effective and clinically relevant in testing potential anti-thrombotic agents. Our adaptation is such that the model was set-up in one femoral artery of the baboons, since the 6B4 Fab fragments do not cross react with canine platelets.

A. Surgical preparation and study protocol

Normal baboons (*Papio ursinus*) weighing 10-15 kg, disease-free for at least six weeks before the experiments, were used. All experiments were approved by the Ethics Committee for Animal Experimentation of the University of the Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa. Baboons were anaesthetized with ketamine hydrochloride (≈ 10 mg/kg IM; Anaket-V, Centaur Laboratory). The intra-arterial pressure was continuously monitored throughout the procedure. Blood for the laboratory tests (examples 3-5) was obtained from one of the femoral veins.

First, a calibrated electromagnetic flow probe was placed around the proximal portion of the isolated femoral artery of the baboons in order to measure arterial blood flow. After the animal was allowed to stabilize for approximately 30 minutes, the endothelium of the femoral artery was injured by gently squeezing with forceps, and cyclic flow reductions due to platelet-dependent thrombus formation were induced by placement of a constrictor. When flow declined to near zero, blood flow through the constricted femoral artery was restored by manually shaking the constrictor. The cyclic pattern of decreasing arterial blood flow following restoration was referred to cyclic flow reductions, and this pattern was continuously monitored for 60 minutes.

The baboons in which the cyclic flow reductions were studied, were divided into three groups. One group (2 baboons) received a placebo (saline solution), the second group (4 baboons) was treated with a bolus injection of 600 µg/kg 6B4 Fab fragments and the third group (3 baboons) received an injection of 2 mg/kg 6B4 Fab fragments. In addition, 4 mg/kg 6B4 Fab fragments were injected in one baboon in order to determine the effect of such high dose on platelet count, receptor occupation, bleeding time and platelet aggregation but cyclic flow reductions were not followed in this baboon.

Animals instrumented to produce cyclic flow reductions were treated with 6B4 Fab fragments or placebo after a 30 minutes baseline monitoring period. Cyclic flow reductions were continuously monitored in each animal during 60 minutes. The anti-thrombotic effect was quantified by comparing the frequency of cyclic flow reductions per hour before and after drug administration. Blood samples for the different laboratory measurements (platelet count, haematocrit, platelet aggregation, receptor occupation and plasma levels) were drawn at several periods in time: before the 60 minutes monitoring period and respectively 30, 60, 150, 300 minutes and 24 hours after treatment.

B. Results

B.1. effect of 6B4 Fab fragments on cyclic flow reductions (Figure 7)

In the baboons that received a placebo injection of saline solution, the frequency of the cyclic flow reductions (CFR) at 60 minutes after injection was not changed (107 ± 7%) significantly as compared to the pre-treatment control

period. A dose of 600 µg/kg 6B4 Fab fragments resulted in a partial inhibition of the cyclic flow reductions, reducing their frequency to 41 ± 15 %. A dose of 2 mg/kg completely abolished the cyclic flow reductions in all three animals studied and this inhibition (6 ± 6%) was observed throughout the 60 minutes study period. Heart rate, blood pressure and haematocrit remained unchanged during the study.

B.2. effect of 6B4 Fab fragments on platelet count and bleeding times (Figures 8 and 9)

The platelet count (Figure 8) was not significantly affected by injection of 600 µg/kg, 2 mg/kg or 4 mg/kg of the 6B4 Fab fragments. Also the bleeding time (figure 9) was not significantly prolonged by injection of 600 µg/kg, 2 mg/kg or 4 mg/kg of the 6B4 Fab fragments.

B.3. Inhibition of ex vivo platelet aggregation (Figure 10)

6B4 Fab fragments inhibited the *ex vivo* ristocetin-induced platelet aggregation in a dose- and time-dependent manner when administered to the baboons (figure 10). Aggregation was totally abolished 30 minutes after injection and, as compared to the aggregation response before injection, aggregation was significantly ($p<0.05$) reduced to 16.8%, 5.2% and 2% 60 minutes and to 68.8% ($p>0.05$), 19.2% and 16% 150 minutes after a bolus injection of 600 µg/kg, 2 and 4 mg/kg 6B4 Fab fragments respectively. The inhibitory effect lasted for about 150 minutes and returned to normal values within 24 hours.

B.4. Receptor occupancy (Figure 11)

The occupancy of GPIb receptors by the 6B4 Fab fragments is shown in Figure 11. Thirty minutes following a bolus injection of 600 µg/kg, 2 and 4 mg/kg 6B4 Fab fragments, approximately 34.5%, 69.3% and 84% of the GPIb receptors were occupied respectively. The receptor occupancy was 28.6%, 64.8% and 79% after 60 minutes; 17.1%, 43.9% and 45.6% after 150 minutes and dropped to 6.3%, 12.9% and 31.3% after 300 minutes following injection of respectively 600 µg/kg, 2 and 4 mg/kg 6B4 Fab fragments. The decrease in receptor occupancy corresponds with the time course of the ex vivo ristocetin-induced aggregation results.

C. Interpretation of experimental results

It is well established that platelet adhesion, activation and aggregation plays a pivotal role in the development of coronary artery syndromes. In particular, the high shear stress present in the constricted coronary arteries is an important initiator of the platelet activation and aggregation. Several investigators have shown that cyclic flow reductions in stenosed damaged canine coronary arteries can be prevented by metabolic inhibition of platelet activation, or by blockade of the GPIIb/IIIa receptor.

In this study we have shown that administration of Fab fragments of the inhibitory anti-GPIb monoclonal antibody 6B4 is effective in diminishing or abolishing cyclic flow variations in stenosed, endothelium-injured femoral arteries in non-human primates. The presumed mechanism by which this occurs is the inhibition of the interaction of the platelet glycoprotein Ib receptor and the vessel wall-bound von Willebrand factor. This prevents platelet activation and aggregation as well as the release of pro-aggregatory and vasoconstrictor substances responsible for these cyclic flow variations.

6B4 Fab fragments completely abolished the cyclic flow reductions at a dose of 2 mg/kg, and reduced them by 59% after injection of 600 µg/kg. Bleeding times were not significantly prolonged, even when injecting 4 mg/kg of the 6B4 fragments, suggesting that 6B4 Fab fragments are a useful anti-thrombotic agent with low bleeding risk. Moreover, there was no fall in platelet count, again indicating that injection of the 6B4 fragments is not expected to cause any haemostatic problems. *In vivo* administration of the 6B4 Fab fragments resulted in a dose- and time-dependent inhibition of *ex vivo* ristocetin-induced platelet aggregation and correlated with the receptor occupancy. The duration of the effects of the 6B4 Fab fragments persisted for about 3 hours when a dose that completely abolished the cyclic flow reductions (>2 mg/kg 6B4 Fab fragments) was given, with receptor occupancy and anti-platelet effects (ristocetin-aggregation) returning to baseline values about 6 hours after injection. In conclusion 6B4 Fab fragments demonstrate the desired properties to be promising compounds for the treatment of acute coronary syndromes with a low bleeding risk.

Example 10 - cloning and sequencing of monoclonal antibody 6B4

In order to reduce the possible immunogenicity of the murine anti-GPIb monoclonal antibody 6B4, it may be necessary to construct chimeric antibodies combining the variable region of the mouse antibody with a human antibody constant region. Depending on the antibody, such chimeric antibodies have been found from substantially reducing to little affecting the immunogenic response. Further humanisation by complementary determining region-grafting or re-surfacing usually has proven to be a successful approach. In order to produce such humanised antibodies, a first step is to determine the sequence of the murine antibody.

Cloning of variable region cDNAs

Total RNA from approximately 3 x 10^7 6B4-hybridoma cells grown in 75 mL T-flasks was prepared using the Qiagen RNeasy Midi Procedure (Westburg) following the manufacturers' instructions and next quantitated by an OD260 reading. cDNA was synthesized from the total RNA by incubating 2 µg of tRNA with 1 µM of poly(dT)$_{15}$ adaptor primer and 4 U of Omniscript reverse transcriptase in a total volume of 20 µl with other reaction buffers and following incubation times as recommended by the manufacturer (Qiagen Omniscript RT Kit) (Westburg).

Next, the V genes were amplified for cloning into the pCRII-TOPO® vector (TOPO TA-Cloning® Kit, In Vitrogen) for sequence determination. Polymerase chain reaction amplification was done using $V_H$back (5'-CAGGTSMARCTGCAGSAGTCWGG-3') and $V_H$for (5'-TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG-3'), $V_L$back (5'-GACATTGAGCTCACCCAGTCTCCA-3') and $V_{K2}$for (5'-GGAAGCTTGAAGATGGATACAGTTGGTGCAGC-3') primers with M,R,W and S respectively (A/C), (A/G), (A/T) and (C/T) (all from Eurogentec, Herstal, Belgium) and $V_H$back, $V_H$for and $V_L$back are complementary to the 5'-terminal part of the framework region FR-1 and to the 3'-terminal part of the FR-4 of the V_H- and V_L-genes respectively and V_{K2} for anneals to the C_K sequence. Polymerase chain reactions were performed in a programmable heating block using 30 rounds of temperature cycling (92°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute). The reactions included the cDNA, 1 µg of each primer and 2.5 U of Hotgold polymerase (Eurogentec) in a final volume of 50 µl, with

the reaction buffers as recommended by the manufacturer (Vitrogen). The polymerase chain reaction product bands were analyzed on a 1.5 % agarose gel.

Transformation was done using the heat-shock method and using *E.coli*-TOP-10 cells (TOPO TA-Cloning® Kit, In Vitrogen) according to the manufacturers' instructions. The cells from each transformation were plated onto LB + ampicillin). Transformation was checked by polymerase chain reaction amplification of the inserts and next analyzed on a 1.5 % agarose gel. Positive clones were grown up for purification of plasmid DNA by the Qiagen Maxi plasmid purification kit.

Sequencing reactions were performed with the ABI Prism Big Dye terminator cycle Sequencing Ready Reaction kit (Perkin Elmer Applied Biosystems, Netherlands) according to the manufacturer's instructions using M13 Forward primer 5'-TTCCTCGACGCTAACCTG-3' and M13 Reverse primer 5'- GATTTAATCTGTATCAGG-3' and which align to the pCRII-TOPO® vector.

Results and interpretation

The cDNA from the heavy-chain variable domain genes were amplified by polymerase chain reaction using primers that hybridize to the framework regions FR-1 and FR-4. For amplification of the light-chain variable domain genes we used a primer that hybridizes to FR-1 and one that anneals in the constant region. These V_H and V_L genes were next cloned into the pCRII-TOPO® vector and transformed into *E.coli* TOP10 cells. By using appropriate primers the V_H and V_L genes were next sequenced. The translated amino acid sequences are given in figures 12 (light chains) and 13 (heavy chains) respectively, and the six complementary determining regions conferring epitope specificity are indicated in these figures. The heavy chain (V_H) revealed a sequence closely related to mouse heavy chain subgroup Ib, whereas the light chain (V_L) gene sequence matches to mouse K-chain subgroup V. Given our choice of priming sites, it is not possible to determine the exact sequence at both ends of the V genes, as it is dictated by the primer (amino acid residues 1-8 of FR1 of the V_L and residues 1-8 of FR1 of the V_H and 111-121 of FR4 of the V_H). Nevertheless, these uncertainties in the

framework regions are unlikely to affect antigen specificity since this is determined by the complementary determining regions.

Table 1.

Platelet counts, plasma levels of 6B4 Fab-fragments, ex vivo ristocetin-induced platelet agglutination and bleeding times following administration of 80-640 µg/kg 6B4 Fab fragments to baboons. Values are given as mean ± SE. Statistical comparisons were made using student t-test for paired sample groups (*p< 0.05).

Dose (µg/kg)	n	Time (min)	Platelet counts (x 10^3/µL)	(% decrease)	Plasma levels (µg/mL)	% inhibition of ex vivo ristocetin-induced (1.5 mg/mL) platelet agglutination	Bleeding times (sec)
0	5	Pre	307 ± 32	(0)	0.07 ± 0.03	0	190 ± 20
80	5	90	272 ± 22	(11)	1.72 ± 0.14	26 ± 9	160 ± 33
160	5	150	248 ± 19	(19)	4.84 ± 0.56	47 ± 12*	250 ± 45
		270	315 ± 31		0.45 ± 0.09	8 ± 3	ND
0	4	Pre	283 ± 23	(0)	0.02 ± 0.01	0	232 ± 42
320	4	90	219 ± 10	(23)	9.13 ± 0.48	25 ± 21	340 ± 63
640	4	150	210 ± 13	(26)	15.35 ± 1.38	80 ± 9*	405 ± 45*
		270	238 ± 20	(16)	1.19 ± 0.09	15 ± 9	ND
		24h	236 ± 13	(17)	0.04 ± 0.01	7 ± 3	ND

ND non determined

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™ LMBP-COLLECTION

Page 1 of Form BCCM™/LMBP/BP/4/99-15 Receipt in the case of an original deposit

Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure

Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the International Depositary Authority BCCM™/LMBP identified at the bottom of next page

International Form BCCM™/LMBP/BP/4/99-15

To : Name of the depositor : Nancy CAUWENBERGHS

Address : K.U. Leuven R & D
Groot Begijnhof
Benedenstraat 59
3000 Leuven

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

NC

I.2 Accession number given by the International Depositary Authority:

LMBP 5108CB

BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™ LMBP-COLLECTION
Page 2 of Form BCCM™/LMBP/BP/4/99-15 Receipt in the case of an original deposit

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II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by:

(mark with a cross the applicable box(es))

- a scientific description — yes ☒ no ☐
- a proposed taxonomic designation — yes ☐ no ☒

III. Receipt and acceptance

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) : August 5, 1999

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM™)
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Vanhoucke

Date : August 23, 1999

Martine Vanhoucke
BCCM™/LMBP curator

CLAIMS

1. A cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB, being able to produce a monoclonal antibody comprising a F_{ab} fragment which binds *in vivo* to human platelet glycoprotein GPIb.

2. A cell line producing a monoclonal antibody having a reactivity identical to that of a monoclonal antibody obtained from the cell line of claim 1.

3. A cell line according to claim 1 or claim 2, wherein the monoclonal antibody F_{ab} fragment further prevents the binding of von Willebrand factor to human platelet glycoprotein GPIb.

4. A cell line according to any of claims 1 to 3, wherein the monoclonal antibody F_{ab} fragment further inhibits platelet adhesion.

5. A cell line according to any of claims 1 to 4, wherein the monoclonal antibody F_{ab} fragment further inhibits platelet activation under high shear conditions.

6. A cell line according to any of claims 1 to 5, wherein the monoclonal antibody F_{ab} fragment further inhibits platelet aggregation under high shear conditions.

7. A F_{ab} fragment, or a homologue having at least 60% amino acid sequence identity therewith, of a monoclonal antibody which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia.

8. A monoclonal antibody F_{ab} fragment or a homologue threreof according to claim 7, which prevents the binding of von Willebrand factor to human platelet glycoprotein GPIb.

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9. A monoclonal antibody F_{ab} fragment or a homologue thereof according to claim 7 or claim 8, which does not produce thrombocytopenia when administered to a primate at a dose of up to at least 4 mg/kg by bolus intravenous administration.

10. A monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 9, which further inhibits platelet adhesion.

11. A monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 10, which further inhibits platelet activation under high shear conditions.

12. A monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 11, which further inhibits platelet aggregation under high shear conditions.

13. A monoclonal antibody comprising a F_{ab} fragment or a homologue thereof according to any of claims 7 to 12.

14. A monoclonal antibody according to claim 13, being produced by on purpose immunization in animals.

15. A monoclonal antibody obtainable from the cell line of claim 1.

16. A monoclonal antibody according to claim 15, being the murine monoclonal antibody 6B4.

17. A monoclonal antibody obtainable from a cell line according to any of claims 2 to 6.

18. A humanized monoclonal antibody derivable from the cell line of claim 1 or from a monoclonal antibody according to claim 15 or claim 16.

19. A humanized monoclonal antibody obtainable from a cell line according to

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any of claims 2 to 6 or from a monoclonal antibody according to claim 17.

20. A pharmaceutical composition comprising a monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 12 in admixture with a pharmaceutically acceptable carrier.

21. A pharmaceutical composition according to claim 20, further comprising a therapeutically effective amount of a thrombolytic agent.

22. A pharmaceutical composition according to claim 21, wherein the thrombolytic agent is selected from aspirin, heparin, tissue plasminogen activators, streptokinase, reptilase and staphilokinase.

23. A pharmaceutical composition according to any of claims 20 to 22, for the prevention or treatment of a haemostasis disorder.

24. A pharmaceutical composition according to any of claims 20 to 23, for oral, intranasal, subcutaneous, intramuscular, intradermal, intravenous, intraarterial or parenteral administration or for catheterization.

25. A monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 12 for use as a medicament.

26. A monoclonal antibody F_{ab} fragment or a homologue thereof according to claim 25, wherein the medicament is for the prevention or treatment of a haemostasis disorder.

27. A monoclonal antibody F_{ab} fragment or a homologue thereof according to claim 25 or claim 26, for simultaneous or sequential association with at least a thrombolytic agent.

28. A monoclonal antibody F_{ab} fragment or a homologue thereof according to claim 27, wherein the thrombolytic agent is selected from aspirin, heparin, tissue plasminogen activators, streptokinase, reptilase and staphilokinase.

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29. A monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 25 to 28, for oral, intranasal, subcutaneous, intramuscular, intradermal, intravenous, intraarterial or parenteral administration or for catheterization.

30. A method of treatment and/or prevention of a haemostasis disorder comprising administering to a patient in need thereof a therapeutically effective amount of a monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 12.

31. A method of treatment and/or prevention according to claim 30, wherein the therapeutically effective amount ranges from 80 µg/kg to 4 mg/kg.

32. A method for the treatment and/or prevention of a haemostasis disorder without inducing thrombocytopenia, comprising administering to a patient in need thereof a therapeutically effective amount of a monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 12.

33. A method of treatment and/or prevention according to claim 32, wherein the therapeutically effective amount ranges from 80 µg/kg to 4 mg/kg.

34. A method according to any of claims 30 to 33, comprising further administration of at least a thrombolytic agent.

35. A method according to claim 34, wherein the thrombolytic agent is selected from aspirin, heparin, tissue plasminogen activators, streptokinase, reptilase and staphilokinase.

36. A method according to claim 34 or 35, wherein the thrombolytic agent is administered simultaneously with the monoclonal antibody F_{ab} fragment or a homologue thereof.

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35 37

37. A method according to claim 34 or 35, wherein the thrombolytic agent is administered sequentially with the monoclonal antibody F_{ab} fragment or a homologue thereof.

38. A polynucleotide encoding for an antigen-binding monoclonal antibody F_{ab} fragment or a homologue thereof according to any of claims 7 to 12.

39. A DNA probe for detecting the polynucleotide sequence of claim 38, comprising a nucleic acid molecule having a sequence complementary to the coding sequence of said polynucleotide.

40. A polynucleotide sequence as shown in SEQ.N°1.

41. A polynucleotide sequence as shown in SEQ.N°2.

42. An amino acid sequence as shown in SEQ.N°3.

43. An amino acid sequence as shown in SEQ.N°4.

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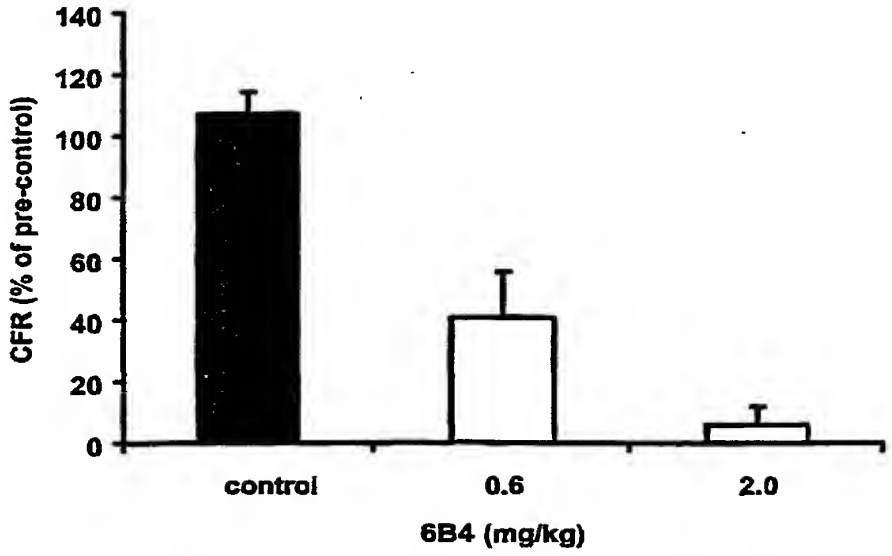
Published:
— *With international search report.*

[Continued on next page]

(54) Title: CELL LINES, LIGANDS AND ANTIBODY FRAGMENTS FOR USE IN PHARMACEUTICAL COMPOSITIONS FOR PREVENTING AND TREATING HAEMOSTASIS DISORDERS



(57) Abstract: A ligand derived from, e.g. a Fab fragment of, a monoclonal antibody obtainable from the cell line deposited with the Belgian Coordinated Collections of Micro-organisms under accession number LMBP 5108CB binds to the human platelet glycoprotein GPib and prevents the binding of von Willebrand factor to said GPIb without inducing thrombocytopenia. The said ligand is useful, in admixture with a pharmaceutically acceptable carrier, in a pharmaceutical composition, optionally further comprising a thrombolytic agent, for preventing and/or treating haemostasis disorders.

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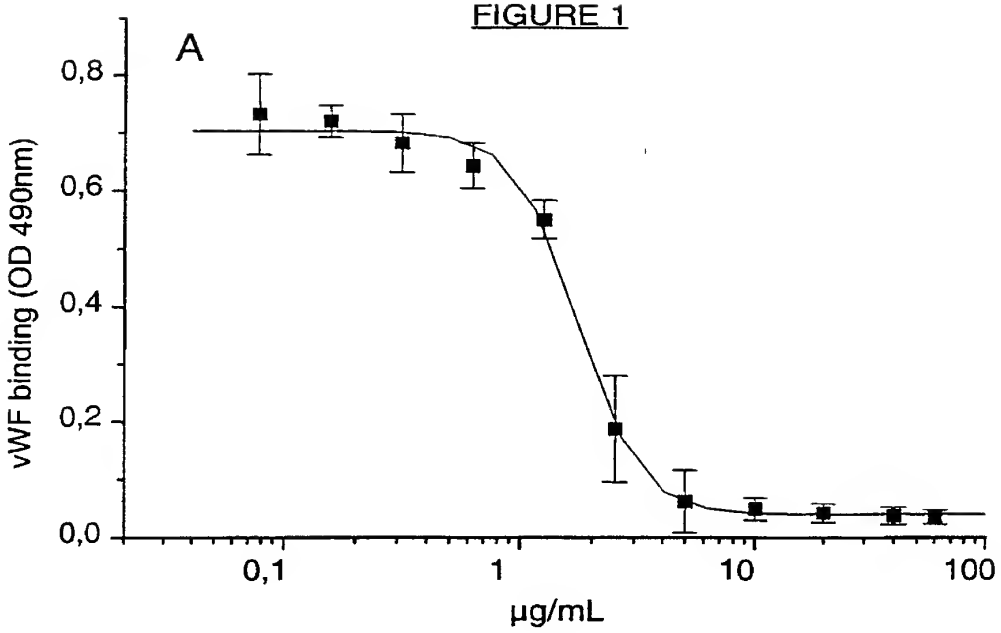
FIGURE 1


A
vWF binding (OD 490nm)
0,0
0,2
0,4
0,6
0,8
0,1
1
10
100
µg/mL

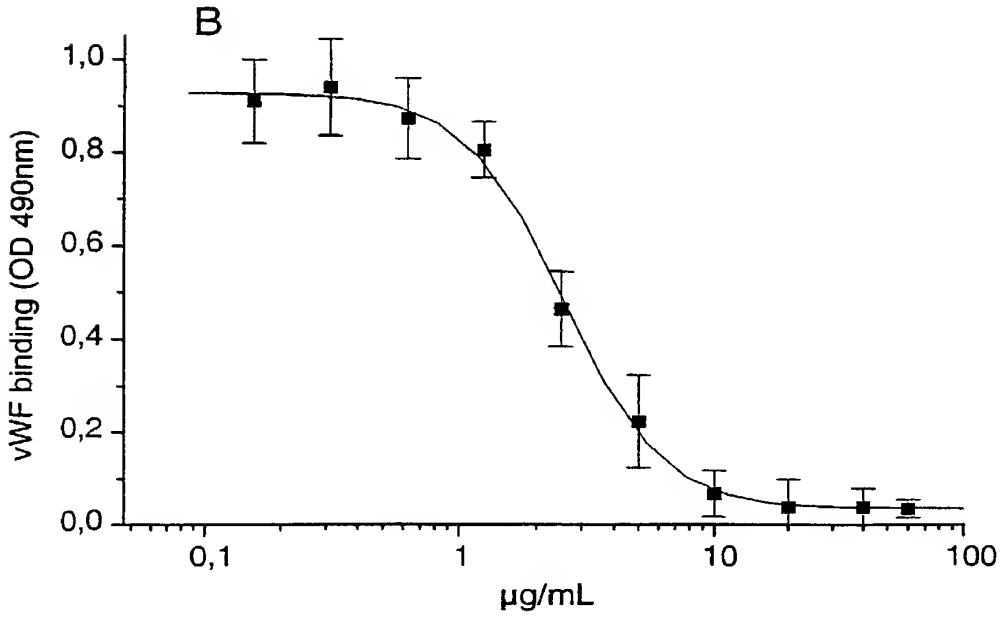

B
vWF binding (OD 490nm)
0,0
0,2
0,4
0,6
0,8
1,0
0,1
1
10
100
µg/mL

FIGURE 2

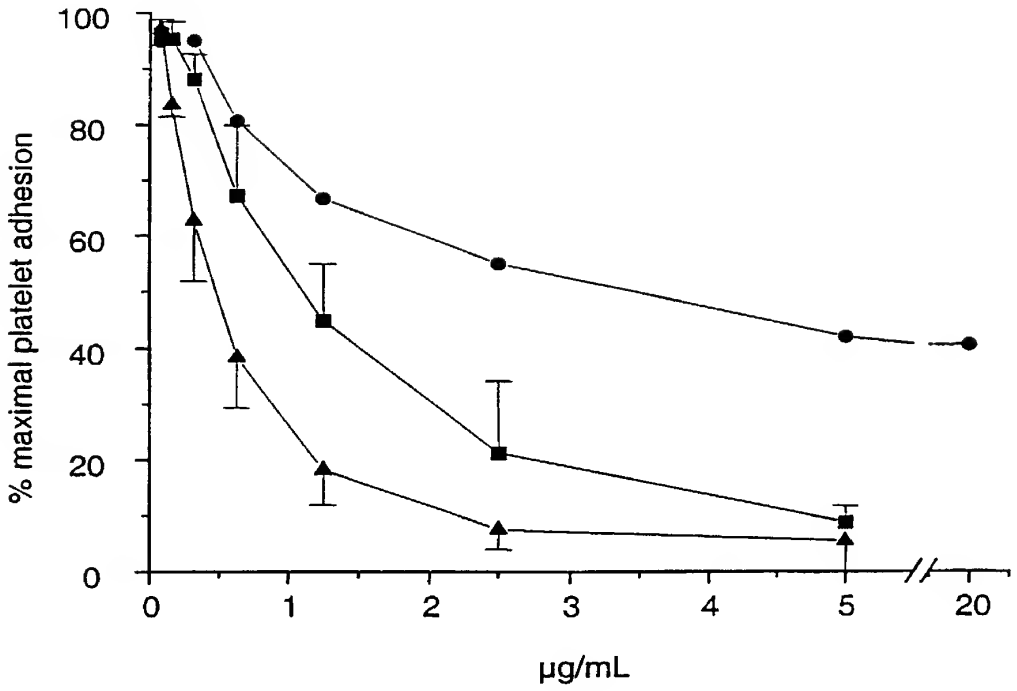


FIGURE 3

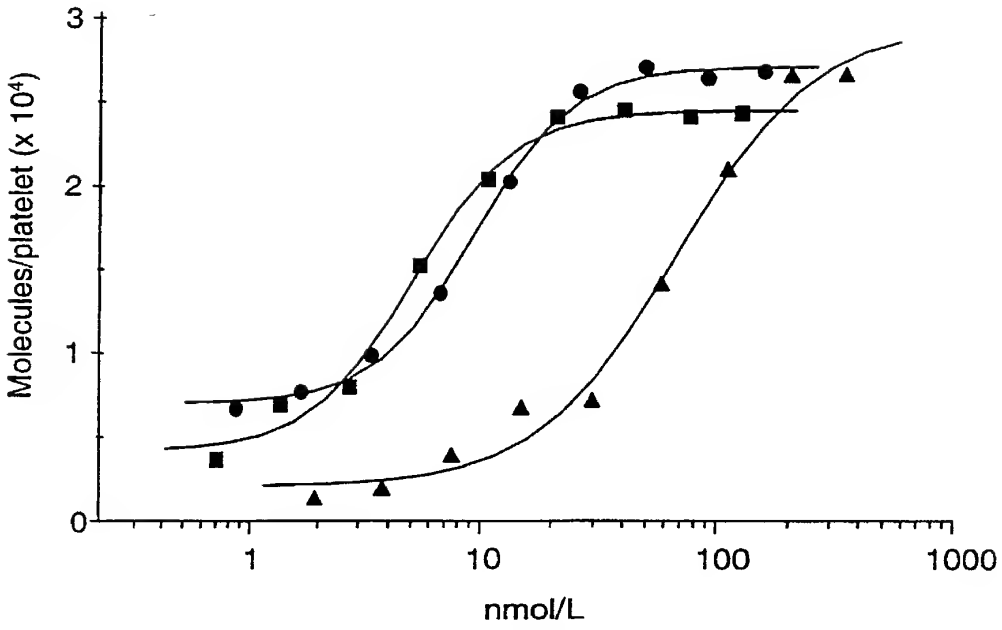


FIGURE 4

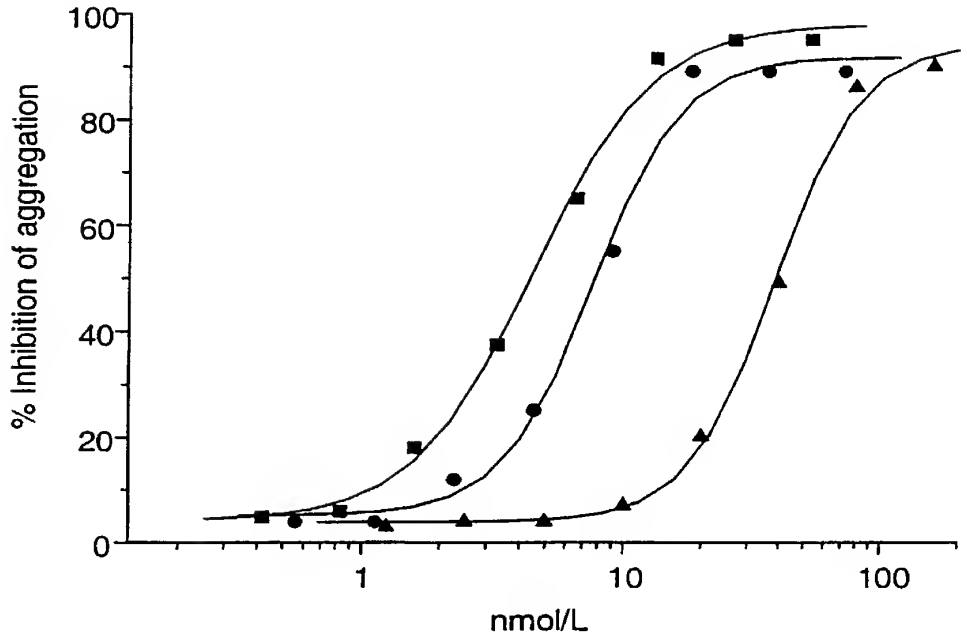
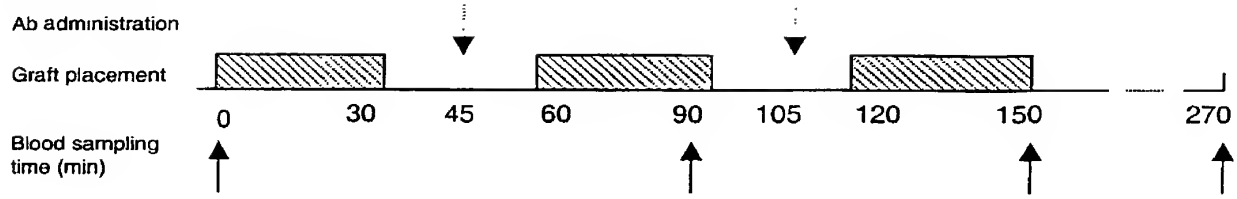
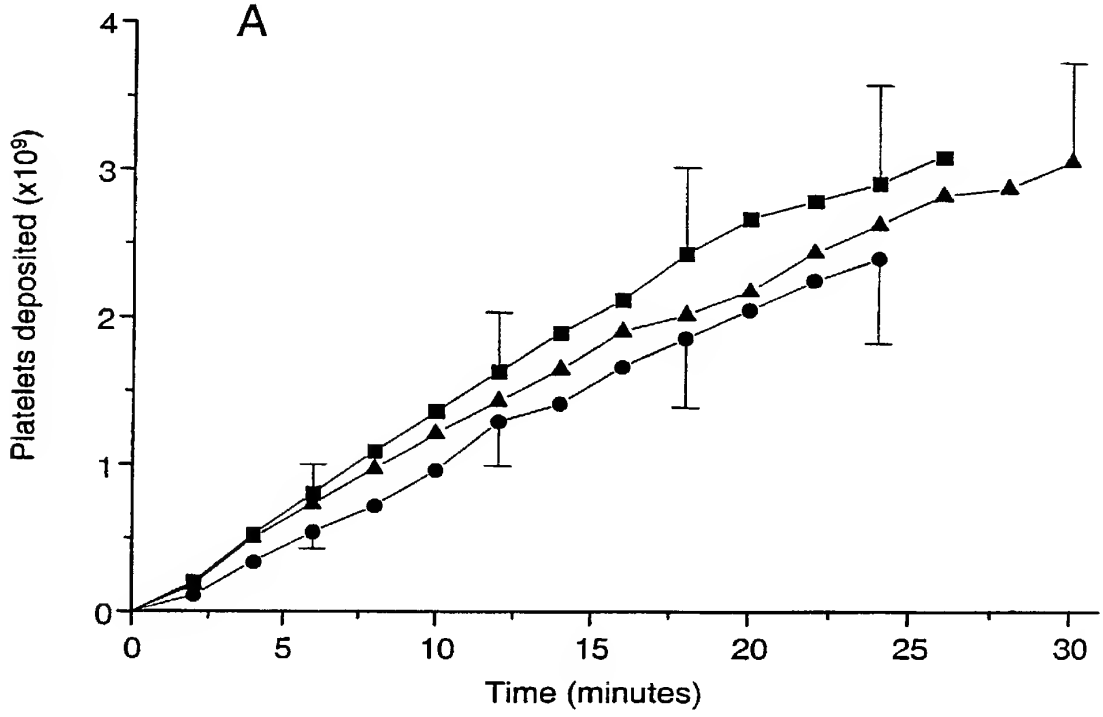


FIGURE 5


Ab administration
Graft placement
0
30
45
60
90
105
120
150
270
Blood sampling time (min)


A
Platelets deposited (x10⁹)
0
1
2
3
4
0
5
10
15
20
25
30
Time (minutes)

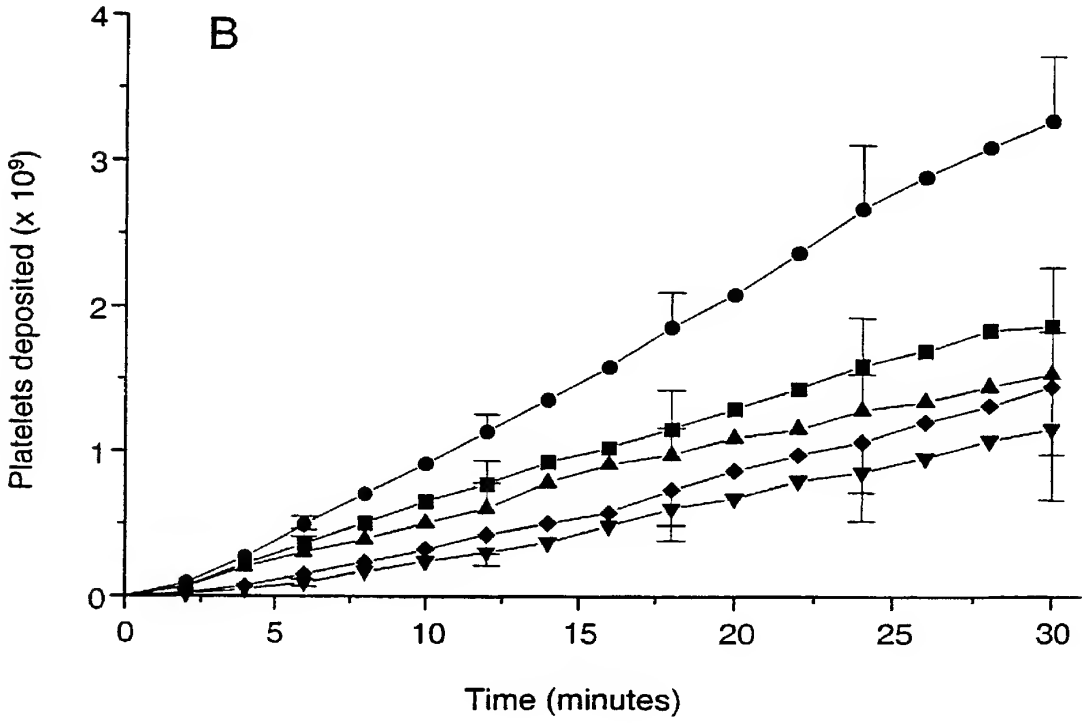

B
Platelets deposited (x 10⁹)
0
1
2
3
4
0
5
10
15
20
25
30
Time (minutes)

FIGURE 6

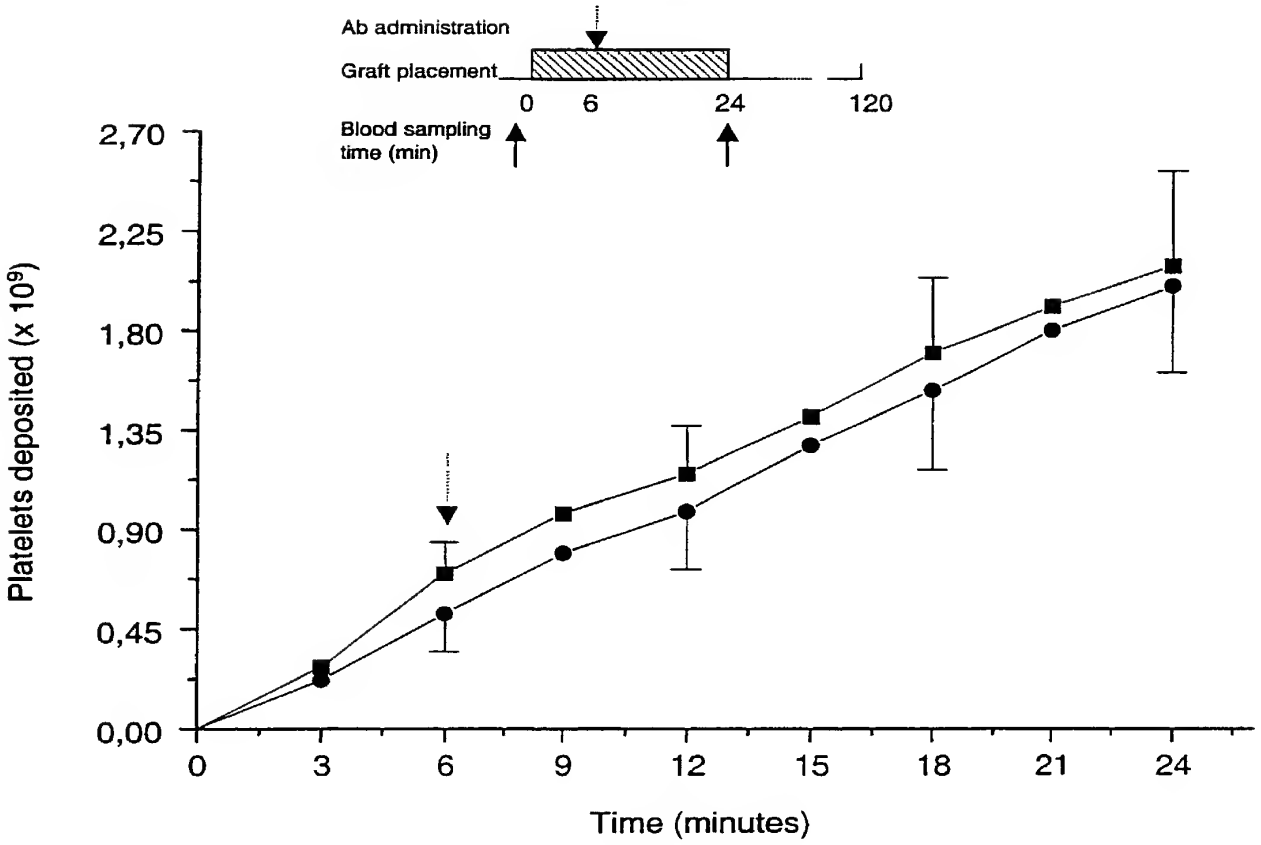
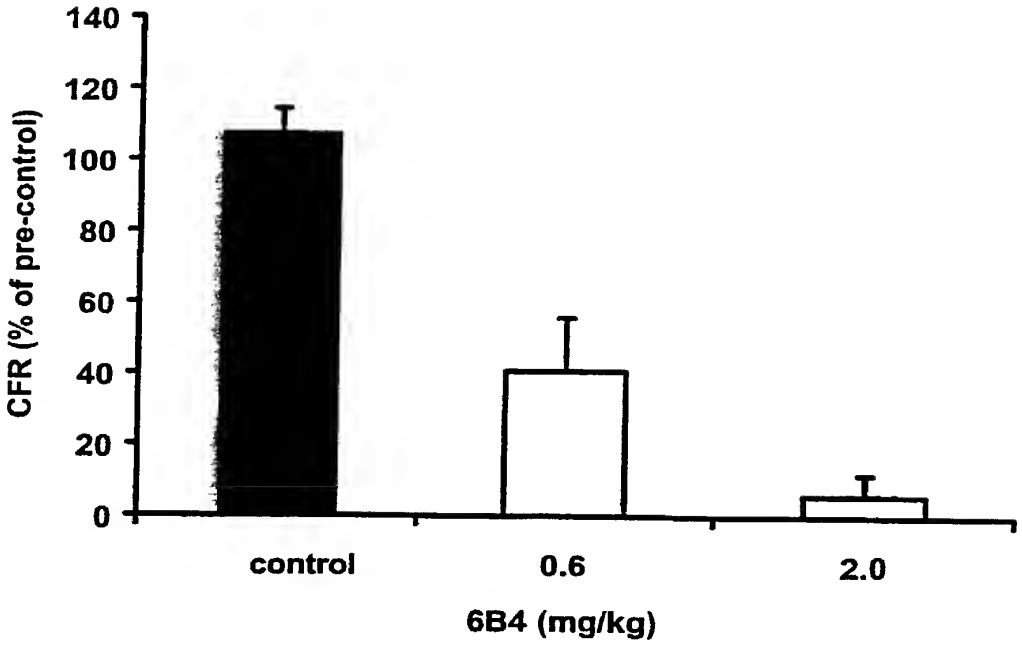

Ab administration
Graft placement
0
6
24
120
Blood sampling time (min)
Platelets deposited (x 10⁹)
2,70
2,25
1,80
1,35
0,90
0,45
0,00
0
3
6
9
12
15
18
21
24
Time (minutes)

FIGURE 7



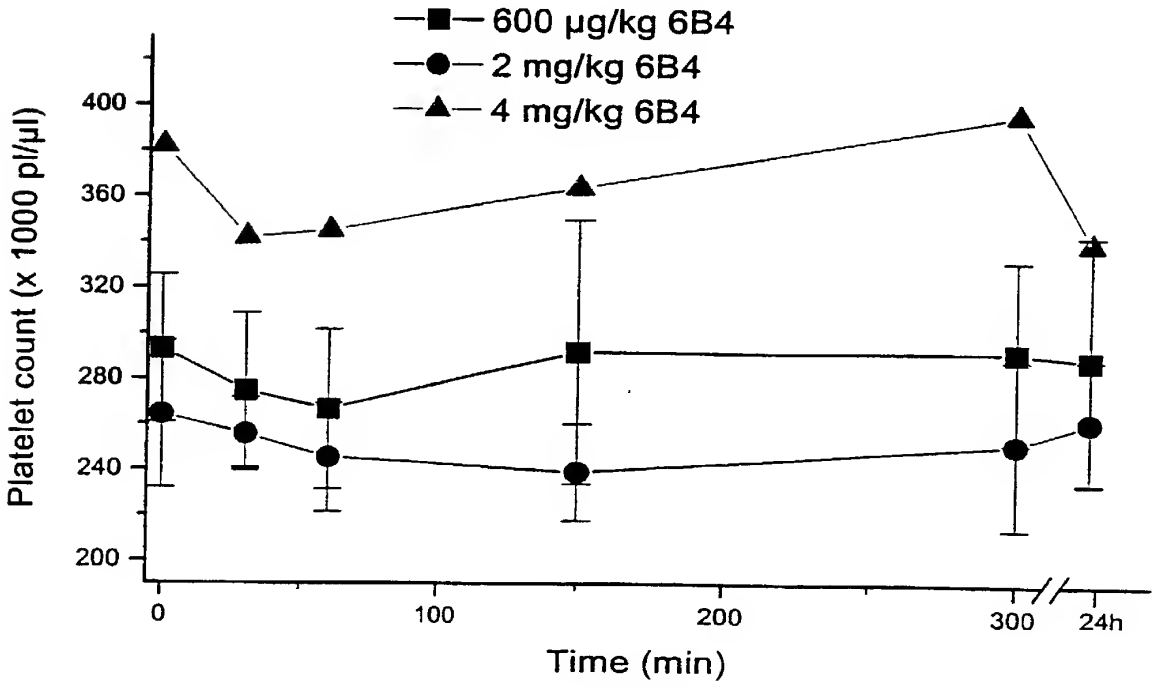
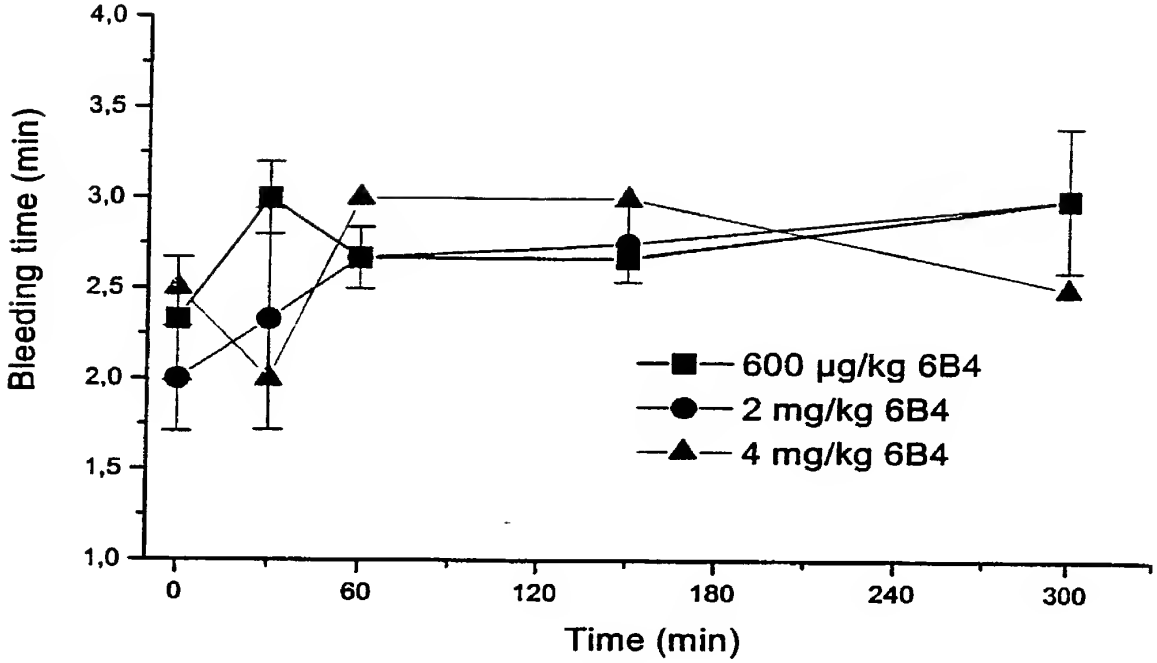


FIGURE 8

FIGURE 9



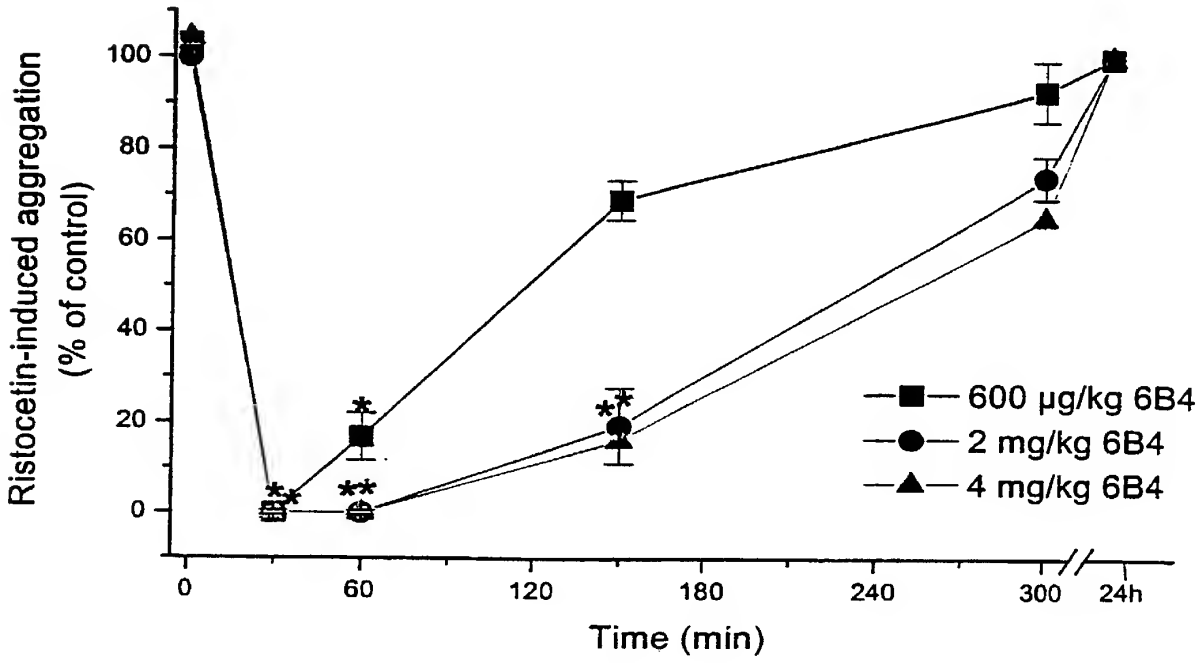


FIGURE 10

FIGURE 11

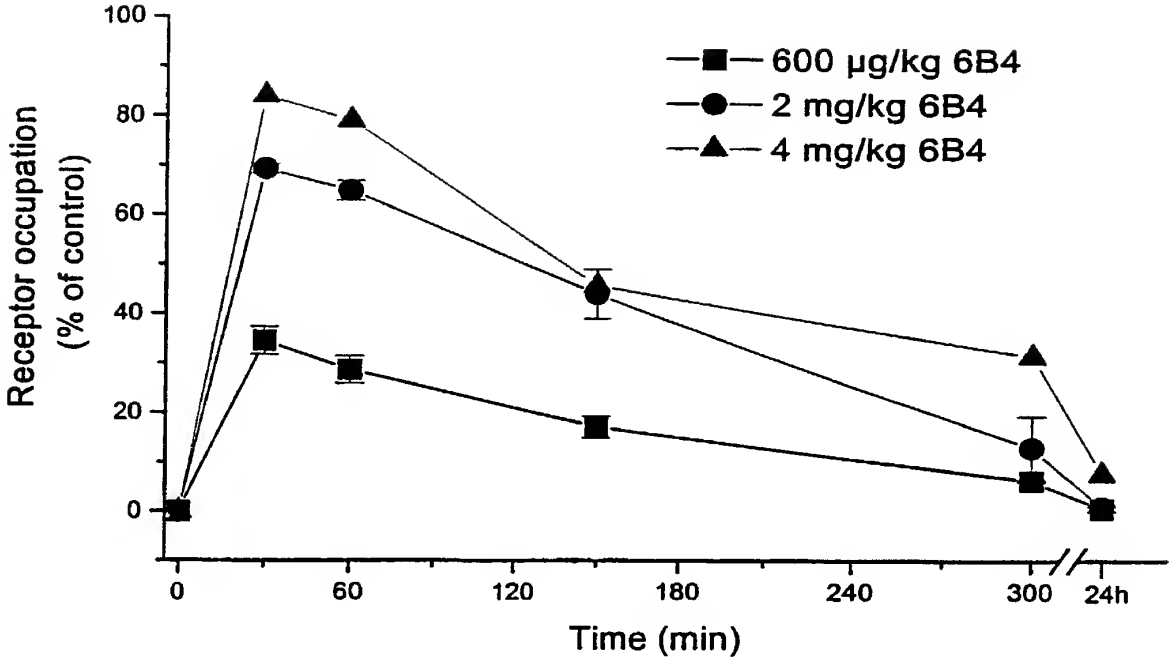

Receptor occupation
(% of control)
100
80
60
40
20
0
600 µg/kg 6B4
2 mg/kg 6B4
4 mg/kg 6B4
0
60
120
180
240
300
24h
Time (min)

FIGURE 12

Nucleic Acid Sequence of 6B4VL

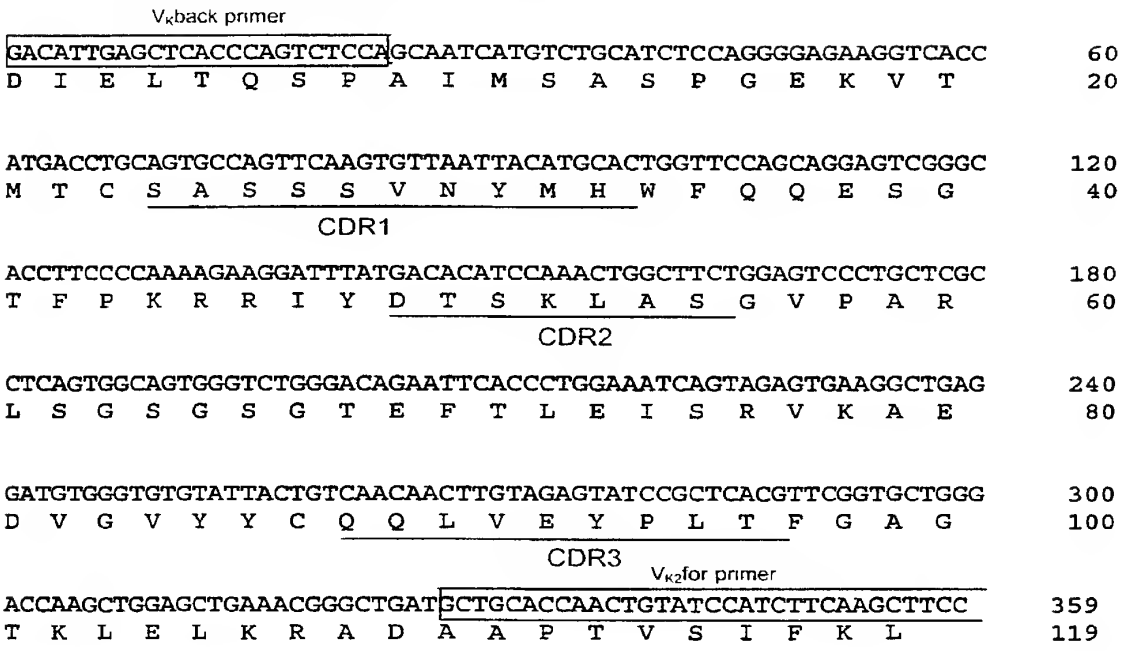


FIGURE 13

Nucleic Acid Sequence of 6B4VH

$V_H$back primer

```
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ACTTGCACTGTCTCTGGGATTTCATTAAACAGATATGGTGTACACTGGGTTCGCCAGCCT     120
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                              CDR1

CCAGGAAAGGGTCTGGAGTGGCTGGGAGTAATATGGACTGGTGGAAGCACAAATTATAAT     180
P  G  K  G  L  E  W  L  G  V  I  W  T  G  G  S  T  N  Y  N       60
                                 CDR2

TCGGCTCTCATGTCCAGACTGAGCATCAGCAAAGACAACTCCAAGAGCCAAGTTTTCTTA     240
S  A  L  M  S  R  L  S  I  S  K  D  N  S  K  S  Q  V  F  L       80

AAAATGAACAGTCTGCAGACTGATGACACAGCCATGTACTACTGTGCCAGAGATCGATCT     300
K  M  N  S  L  Q  T  D  D  T  A  M  Y  Y  C  A  R  D  R  S       100

                                                $V_H$for primer
ACTATGATTACGGCCTATGCTATGGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCC     360
T  M  I  T  A  Y  A  M  D  Y  W  G  Q  G  T  T  V  T  V  S       120
CDR3

TCA     363
S       120
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#5

Attorney Docket No. 522-1778

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Cell lines, ligands and antibody fragments for use in pharmaceutical compositions for preventing and treating haemostasis disorders, the specification of which:

___ is attached hereto.

x was filed on August 8, 2000 as

Application Serial No. PCT/EP00/07874 and

was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

Country	Number	Date Filed	Priority Claimed Yes	No
Great Britain	9918788.2	August 10, 1999	x	
Europe	00102032.0	February 2, 2000	x	

I hereby claim the benefit under Title 35, United States Code Section 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, Section 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No.	Filing Date	Status
PCT/EP0007874	August 8, 2000	Pending

And I hereby appoint William M. Lee, Jr., Registration No. 26,935, Thomas E. Smith, Registration No. 18,243, Dennis M. McWilliams, Registration No. 25,195, James R. Sweeney, Registration No. 18,721, Glenn W. Ohlson, Registration No. 28,455, David C. Brezina, Registration No. 34,128, Jeffrey R. Gray, Registration No. 33,391, Gerald S. Geren, Registration No. 24,528, Timothy J. Engling, Registration No. 39,970, Peter J. Shakula, Registration No. 40,808, Robert F. I. Conte, Registration No. 20,354, Howard B. Rockman, Registration No.

(14)

22,190, John W. Hayes, Registration No. 19,286, and Mark A. Hagedorn, Registration No. 44,731, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith. It is requested that all communications be directed to Lee, Mann, Smith, McWilliams, Sweeney & Ohlson, P.O. Box 2786, Chicago, Illinois 60690-2786, telephone number (312) 368-1300.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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Signature Hans Deckmyn Date 25/02/02

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Country of Citizenship: Belgium

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2-00 BEX

Full name of joint inventor: Nancy Cauwenberghs

Signature [signature] Date 28/02/02

Country of Residence: Belgium

Country of Citizenship: Belgium

Post Office and Residence Address: Heerbaan 130, B-1840 Londerzeel, Belgium

BEK

SEQUENCE LISTING

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Cauwenberghs, Nancy

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